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Recent advances in antiphospholipid syndrome

Edited by
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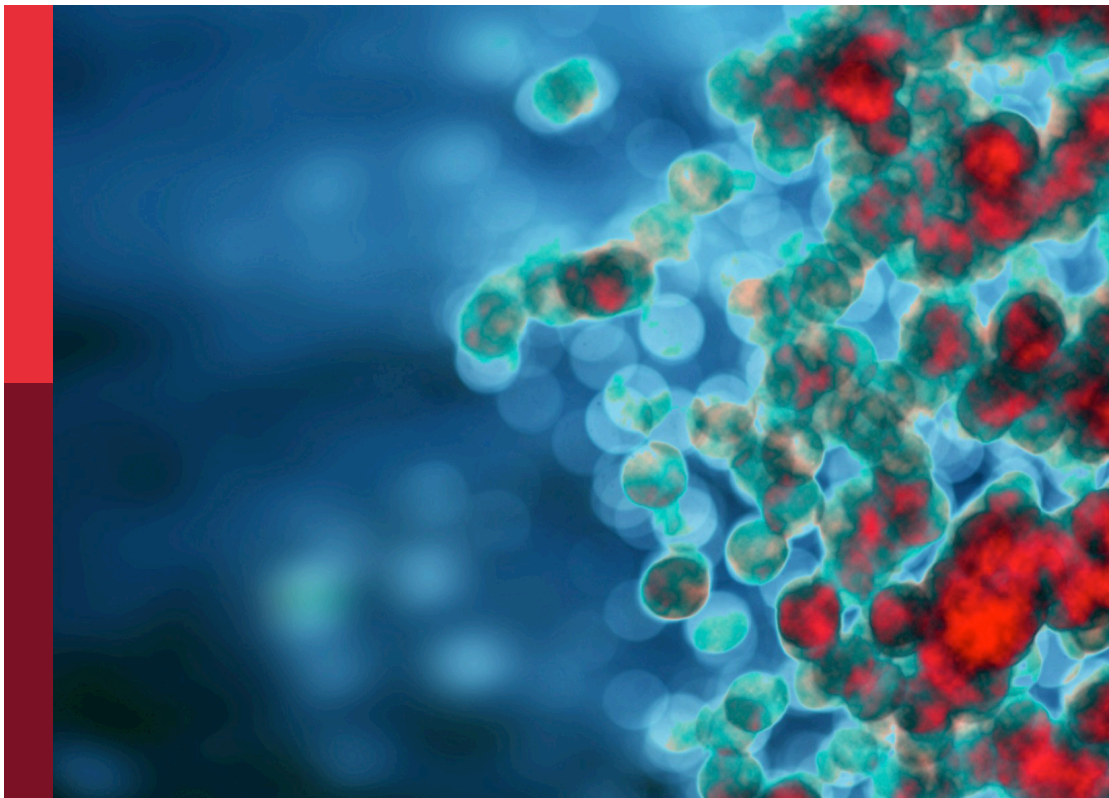

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Recent advances in antiphospholipid syndrome

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CASE REPORT
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Case Report: Resetting the Humoral Immune Response by Targeting Plasma Cells With Daratumumab in Anti-Phospholipid Syndrome

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Introduction: Monoclonal antibodies (mAb) targeting plasma cells are malignant gammopathy designed and approved therapies. In recent years, these antibodies have also been increasingly introduced for non-malignant conditions such as autoimmune-mediated diseases. The Anti-Phospholipid Syndrome (APS) is an immune-mediated disorder in which autoantibodies against phospholipid associated proteins could elicit the activation of the coagulation cascade in specific situations. Therefore, the mainstream treatment for APS patients is the use of anticoagulant therapy. However, there are refractory patients who would benefit from targeting the antibodies rather than their effects. Rituximab, a B-cell depleting mAb, and intravenous immunoglobulins (IVIG) have been used in APS patients without showing a clear beneficial effect or a significant drop in anti-phospholipid antibody (aPL) levels.

Clinical case: We present our first APS case treated with daratumumab, an anti-CD38 mAb, in a 21-year-old patient with APS who presented with recurrent venous thromboembolic events despite adequate anticoagulant therapy. She tested positive for lupus anticoagulant, anti-cardiolipin IgG, anti-beta-2-glycoprotein-I IgG and anti-phosphatidylserine/prothrombin IgG and IgM. She was administered one dose weekly of daratumumab for 4 weeks. The treatment showed an adequate safety profile and was well tolerated. The patient was discharged after undergoing a clinically significant improvement. After the therapy, her levels of positive aPL declined significantly and most continued to decrease during the next three months. The patient experienced a new thrombotic episode two years after the therapy associated with poor adherence to antithrombotic therapy.

Conclusions: The treatment with daratumumab showed an adequate safety profile, was well tolerated and led to a significant clinical improvement. Levels of aPL lowered on therapy and the next three months and then rose again during follow-up. Further investigation is needed to better elucidate the role and optimal timing and doses of daratumumab in treatment of refractory APS.

Keywords: anti-phospholipid, refractory, treatment, daratumumab, anti-CD38

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by vascular thrombosis and/or pregnancy morbidity in the presence of persistent antiphospholipid antibodies (aPL) (1). Antibodies against beta-2-glycoprotein I (aB2GPI) and Cardiolipin (aCL), together with the functional assay lupus anticoagulant (LA), are the three laboratory tests considered for classification of this Syndrome in the revised Sydney criteria (1).

Since thrombosis is the most relevant clinical manifestation of this disorder, its treatment relies almost exclusively on anticoagulant therapy with warfarin, acenocoumarol and heparin-based regimens. EULAR recommendations from 2019 suggest assessing the anticoagulation range using International Normalized Ratio (INR)) (2). If the INR is found to be lower than the target, the patient would need the anticoagulant therapy to be adjusted to maintain the INR within the therapeutic ranges (INR: 2–3) (3). If the INR is within the therapeutic ranges at the time of a thrombotic episode, it is recommended to increase the intensity of anticoagulation to INR: 3–4 (2, 3). In patients with arterial events, another option would be to add low-dose acetylsalicylic acid (LDASA) to the anticoagulant treatment. However, it should be noted that this combination is burdened by a higher bleeding risk (4). Long-term low molecular weight heparin (LMWH) may also be considered as a safe and effective alternative to warfarin, according to the guidelines from the 13th International Congress on Anti-Phospholipid Antibodies (5–9). However, despite anti-thrombotic therapy, 3 to 24% of APS patients still develop recurrent thrombotic events (10–13).

Patients with proven refractoriness to the anticoagulant therapy would benefit from etiological targeted therapies, as aPL are both diagnostic markers and pathogenic drivers of APS (14). Consequently, the 15th International Congress on Anti-Phospholipid Antibodies Task Force on Treatment Trends suggested that immunomodulatory therapy, in addition to or as an alternative to oral anticoagulation, could represent a valuable option for refractory cases (15).

Much of the accumulated experience on the use of immunomodulatory treatments in APS comes from the need to treat refractory patients. Some of them suffer from the catastrophic form of APS but most are carriers of conventional APS with persistent additional symptoms such as migraine, livedo reticularis, and fatigue, which are not included in the latest classification criteria. Most immunomodulatory agents used in APS usually target several steps in B-cell differentiation pathway and their activity. For example, corticosteroids difficult B-cell proliferation and maturation by inhibiting NFkB pathway (16). Other drugs, like hydroxychloroquine, show anti-inflammatory and anti-thrombotic effects (17) by detaching aPL from B2GPI on phospholipid-bound endothelial membranes and protecting annexin V (12). Plasmapheresis removes pathogenic mediators from blood such as autoantibodies and cytokines (18). Intravenous immunoglobulin (IVIG) helps blocking autoantibodies and autoreactive B-cell activation and expansion (19). Among them, hydroxychloroquine is one of the most largely prescribed. It has shown beneficial effects for secondary thromboprophylaxis, especially in SLE patients (20, 21). The combination of glucocorticoids, plasmapheresis or Intravenous immunoglobulins (IVIG) have also shown to be effective in preventing recurrent thrombosis in catastrophic (22) and non-catastrophic APS patients (23). While IVIG is preferred in APS patients with immune thrombocytopenia, plasmapheresis is recommended in microangiopathic hemolytic anemia. Eculizumab, a monoclonal antibody (mAb) blocking complement C5a, has been used for secondary thromboprophylaxis in kidney transplant patients with APS (24). Rituximab, a chimeric mAb targeting CD20 on the surface of B cells, was studied in an open-label phase IIa descriptive pilot study (RITAPS) carried out in 20 patients with non-criteria APS manifestations refractory to conventional treatments (25). The drug proved to be effective in controlling some of symptoms, but levels of aPL remained unchanged (25).

Most of the above-mentioned drugs target B-cell activation, proliferation and/or lower the concentration of circulating antibodies for a short period of time, but they are not directed against the main sources for antibody secretion, which are known to be bone marrow-resident plasma cells (26, 27). These cells express the surface marker CD38 and have the ability to continuously secrete antibodies without further stimulation by their cognate antigen for years or decades. In autoimmunity, as autoreactive B-cells were supposed to be persistently activated by contact with self-antigens, short-lived plasma cells or plasmablasts, which are proliferating cells with a life span of 3 to 5 days (28), were thought to be the most relevant source for autoantibody production. Nonetheless, the clinical experience has proved this hypothesis to be wrong. Treatment with dexamethasone or cyclophosphamide causes a significant reduction in the presence of plasma cells in inflamed tissues, but not in bone marrow niches (27). These untargeted cells continue producing pathogenic autoantibodies that could form immune complexes and reactivate the disease *via* induction of inflammatory pathways in antigen presenting cells or motivate a direct activation of autoreactive memory B-cells (27). Our group previously described the association of thrombosis (29, 30) and non-criteria manifestations (31) in patients with APS and circulating immune complexes with B2GPI.

As the CD38 molecule is expressed on the surface of plasmablasts and plasma cells, although not exclusively (e.g.: on NK cells), they are targeted by daratumumab, an anti-CD38 IgG1-kappa human mAb. The binding of daratumumab to CD38 molecules on the surface of cells raises antibody-dependent cellular cytotoxicity and the depletion of the cells (32). Daratumumab was approved in 2016 in combination with lenalidomide and dexamethasone, or bortezomib and dexamethasone, for the treatment of multiple myeloma patients as a second line therapy (33). However, some off-label uses have been reported in the literature for autoimmune hemolytic anemia (34–37), Evans' Syndrome (38, 39), cold agglutinin disease (40, 41), pure red cell aplasia (42), immune thrombocytopenia (43), systemic lupus erythematosus (44), and anti-CASPR2 encephalitis (45).

We present the case of a young woman diagnosed of Primary APS with recurrent venous thromboembolic events, in whom the

failure of conventional treatment and plasmapheresis led to off-label use of daratumumab.

METHODS

Patient Consent and Approval of Off-Label Use of Daratumumab

Off-label use of daratumumab was approved by the Medical Direction and the Pharmacy Department of our center. The patient was informed about the goals and possible side effects of the proposed treatment with daratumumab and signed the informed consent. The treatment was funded by our center.

Blood Samples

Fresh blood samples were drawn from the patient whenever she came to the Hospital and before/after a relevant time point regarding the administered therapy.

Determination of Anti-Phospholipid Antibodies

The presence of aCL and aB2GPI IgG/IgM was evaluated using a Phadia 250 (ThermoFischer Scientific, MA, USA) with cutoffs established by the manufacturer and validated in our laboratory (99th percentile). LA was measured using HemosIL dRVVT (cutoff ratio 1.2) and HemosIL Silica Clotting Time (cutoff ratio 1.3) assays (Instrumentation Laboratory SpA, Milano, Italy). aPS/PT IgG/IgM antibodies were evaluated using QUANTA Lite ELISA (INOVA DIAGNOSTICS, San Diego, CA, USA) with cutoffs established by the 99th percentile of our local healthy population (IgG: 30 U/mL and IgM 40 U/mL).

Total Serum Immunoglobulin Quantification

Total serum IgG, IgA, and IgM were evaluated using an Immage 800 nephelometer (Beckman Coulter Fullerton, CA, USA).

Lymphocyte Subpopulations Study

Lymphocytes were labeled using whole blood (50 μL) and 20 μL of BD multitest 6-color TBNK reagent in Trucount tubes for 15 min. Red blood cells were lysed using fluorescence activated cell sorting lysing solution. Plasmablasts were assessed by a combination of monoclonal antibodies as CD45+CD19+CD38+++IgM− lymphocytes. Determination of lymphocyte subpopulations was performed with a FACSCanto II flow cytometer, and data analyzed by FACSCanto clinical software (BD Biosciences, San Jose, CA, USA).

RESULTS

Case Description and Follow-Up

A previously healthy 19-year-old woman taking oral contraceptives was diagnosed of right pulmonary thromoembolism (PTE). As other risk factors such as obesity, smoking or inherited thrombophilia were ruled out, she was investigated for aPL and resulted in positive titers of LA, aCL IgG, aB2GPI IgG and anti-phosphatidylserine/prothrombin (aPS/PT) IgG and IgM. Anti-nuclear antibodies (ANA), complement C3 or C4 consumption or other clinical signs associated to SLE were absent. She was diagnosed of Primary. APS and discharged with acenocoumarol and hydroxychloroquine. Two years later, she developed a new PTE despite adequate INR target. Acenocoumarol was discontinued, and treatment with Low Molecular Weight Heparin (LMWH) and low doses of aspirin were initiated. Four weeks later she was admitted again because of severe left iliofemoral vein thrombosis (VT).

On admission, we switched LMWH to parenteral sodium heparin with well-documented therapeutic anticoagulant activity. On day 1 she began with dyspnea and desaturation. A pulmonary angioCT scan demonstrated the presence of PTE. On day 2, she worsened clinically, and a new angioCT scan demonstrated the growth of the pulmonary thrombus.

To address this worsening clinical picture, we planned a sequential treatment regimen based on two-dose plasmaphereses (days −9 & −8 before the start of treatment with daratumumab) with albumin as reposition, 20 g IVIG (day −4) as a single replacement therapy after plasmapheresis and 4 doses of anti-CD38 daratumumab on days 0, + 7, +14 & +20. The first dose of daratumumab consisted of 8 mg/kg. In the second, third and fourth infusions, 16 mg/kg were administered. The patient experienced a clear improvement of symptoms and signs of VT and PTE from day +1 and could be discharged later on with a therapeutic regimen of subcutaneous enoxaparin (day +8). The overall tolerability of the treatment was adequate. She only developed mild diarrhea with the infusions and one upper respiratory tract infection.

She showed a partial response in her blood analyses from the start of the anti-CD38 therapy with all positive aPL slowly declining. Positive aPL reached their lowest values on day 108 after the first infusion of the drug and 84 days after the end of treatment. Since then, aCL IgG, aB2GPI IgG and aPS/PT IgG began a smooth increase until the time of this report, with aPL values surpassing those from baseline (**Figure 1**). However, aPS/PT IgM values have remained stable on a plateau at half the titer of baseline values.

Almost two years later, and coinciding with the rise of aB2GPI and aCL of IgG isotype, the patient unintentionally missed 6 doses of enoxaparin and was admitted to Hospital with a new VT. Since that episode, she has remained asymptomatic in the follow-ups with an adjusted dose of subcutaneous enoxaparin every 12 hours.

Besides aPL, changes in the total serum immunoglobulins were noted, with a slow decline in total IgG until 4.58 g/L. To avoid susceptibility to infections, the patient was administered a single dose of 60 g polyclonal IVIG on day +79 and has remained within normal levels during the follow-up. Lymphocyte counts did not change significantly but a decline and absence of circulating NK cells was noted after the first infusion. NK count was restored two months after the end of the therapy. As expected by their surface expression of CD38, plasmablasts were removed from peripheral blood and did not show signs of

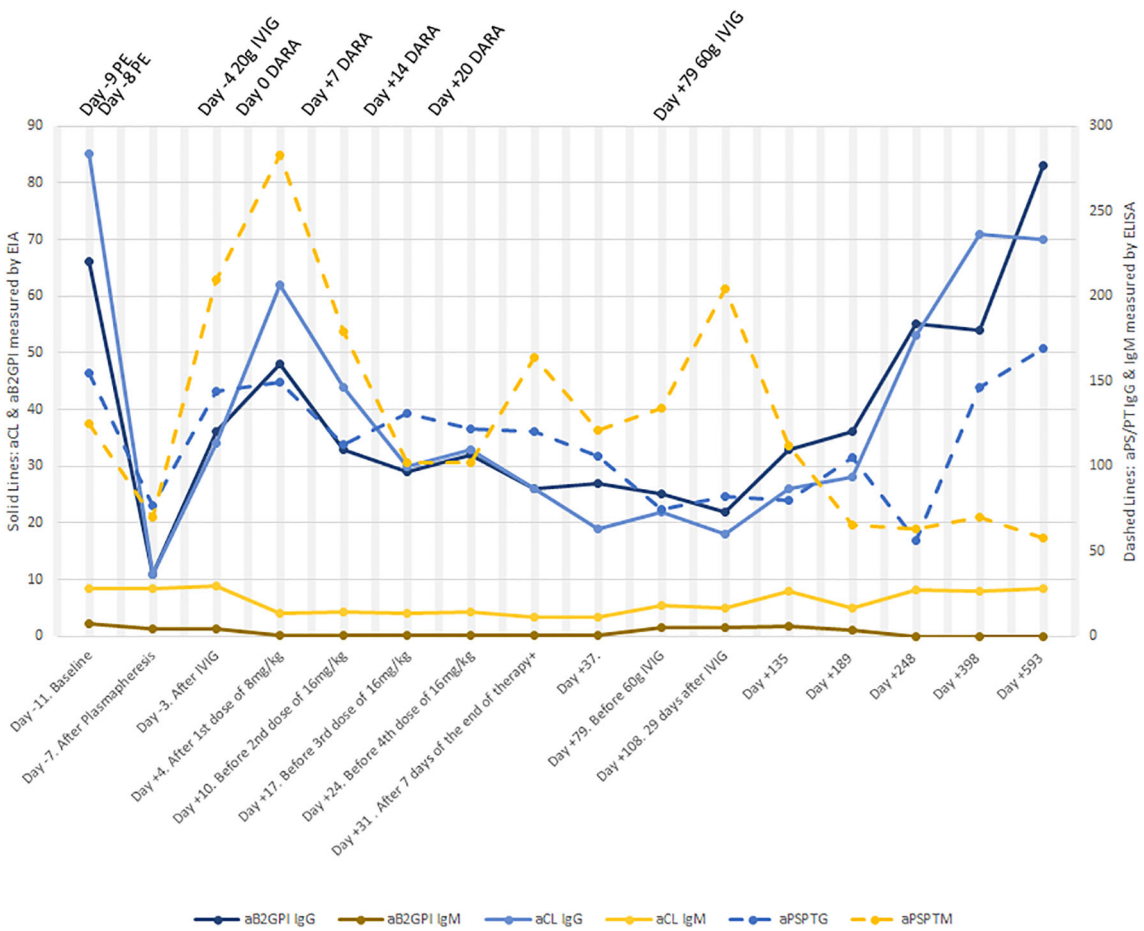

FIGURE 1 | Change in aPL levels before and after therapy. An extreme drop in aPL levels lasting for 4 days was observed after two sessions of plasmapheresis. aPS/PT IgM values even rose to higher levels than in baseline after plasmapheresis. One week after the first dose of daratumumab was administered, aCL IgG and aB2GPI IgG started a decline, this decline lasting until day +108. After day +108, a significant climb began. Levels of aPS/PT IgG lowered until day +248 and then went up again. Levels of aPS/PT IgM showed an intermittent up and down profile, but they have remained stable at half the titer of baseline from day +189 and onwards. PE, plasmapheresis; DARA, daratumumab.

restoration until eight months after the end of therapy. Other B-cell subpopulations did not change significantly (**Table 1**).

DISCUSSION

As far as we know, this is the first reported use of anti-CD38 therapy in a patient with APS. This treatment was aimed at target plasma cells under the hypothesis that aPL are not only produced by memory B cells but also by bone marrow resident plasma cells. The decision of whether to give rituximab or daratumumab, both off-label uses, for this young lady with repeated PTE and VT despite right anticoagulant therapy, balanced in favor of daratumumab after a careful reading of the evidence. All the studies we found in the literature showed no clear changes in aPL levels after rituximab therapy. Targeting plasma cells also made sense as these cells play an important role in the pathogenesis of autoimmune diseases such as SLE (44, 46), systemic sclerosis (47), Sjögren syndrome (47), ANCA-vasculitis (47), autoimmune cytopenia (34), and rheumatoid arthritis (46).

In our patient, we used a conservative treatment scheme with one dose of 8 mg/kg and three of 16 mg/kg separated by 1 week, with a total duration of the cycle of 1 month. As anti-CD38 mAb therapy was initially developed to kill malignant plasma cells, at the time of administration, we did not have the experience to suggest that a shorter or longer cycle would be effective. At the time of writing of this manuscript, off-label use of daratumumab for autoimmune diseases has increased and some cases have been reported in the literature. In the most recent cases, the authors chose longer cycles with more doses than we proposed for our patient (41).

Clinically, the patient improved from a previously dramatic scenario in which continuous heparin infusion was administered without successful control of the thromboembolic disease.

As a result of the combined interventions made which included two consecutive sessions of plasmapheresis and

TABLE 1 | Levels of aPL, serum immunoglobulins, d-dimer and lymphocyte subpopulations.

Time Point	aPS/PT IgG	aPS/PT IgM	aB2GPI IgG	aB2GPI IgM	aCL IgG	aCL IgM	Total Serum IgG	Total Serum IgA	Total Serum IgM	D-Dimer	Total Lymphocyte Count	NK %	NK	CD19%	CD19	Plasmablasts%
Day –11. Baseline	155	125	66	2.1	85	8.5	8.8	0.9	2.1							
Day –7. After Plasmapheresis	77.1	69.9	11	1.4	11	8.4	3.2	0.5	0.6		**1418**	6.1	87	27.4	389	
Day –3. After IVIG	144	210	36	1.4	34	8.9	10.5	0.8	1	2028						
Day +4. Right after 1st dose of 8 mg/kg	149	283	48	0.1	62	4.1	10.1	1.1	1.5	3497	1631	0.3	5	35.2	575	0.3
Day +10. Before 2nd dose of 16 mg/kg	113	179	33	0.1	44	4.3	7.8	0.5	0.9	878	1656	1.7	28	26.9	446	
Day +17. Before 3rd dose of 16 mg/kg	131	102	29	0.1	30	4.1	6.8	0.3	0.8	397	1728	0.7	12	27.2	470	
Day +24. Before 4th dose of 16 mg/kg	122	102	32	0.1	33	4.3	6.1	0.2	0.6	178	1660	**0.3**	**4**	30	498	
Day +31. After 7 days of the end of therapy	120	164	26	0.1	26	3.4	6.9	0.2	0.8	320	1628	1.2	20	29.1	473	
Day +37.	106	121	27	0.1	19	**3.3**	6.2	0.1	0.7	340	1606	0.8	12	24	386	**0**
Day +79. Before 60 g IVIG	74.2	134	25	1.5	22	5.4	**4.5**	**0.1**	**0.7**	**264**	3116	3.4	108	15.2	475	**0**
Day +108. 29 days after IVIG	82.2	204	**22**	1.6	**18**	5	11.5	0.1	1.1	610	2189	2.6	58	20.1	439	0.1
Day +135	79.6	112	33	1.8	26	8	8.5	0.1	1.1	1280	3069	3.8	117	15.4	475	
Day +189	105	65.5	36	1.1	28	5	6.6	0.2	1.2	201	2500	3.9	98	14.4	362	
Day +248	**56.1**	63.1	55	0.1	53	8.1	6.8	0.3	1.5	563	2003	4.7	95	16	320	0.6
Day +398	146	69.7	54	0.1	71	8	5.9	0.4	1.9	1855	2330	4.4	101	19	442	
Day +593	169	**58.1**	83	0.1	70	8.5	5.7	0.4	1.4	2348	2541	2.9	74	27.7	704	

Bold values represent the lowest levels achieved for every parameter. Besides aPL levels variation, which is described in ***Figure 1****, all serum immunoglobulins were affected after treatment with daratumumab, with IgG showcasing the most relevant decline. D-dimer also lowered after treatment and then went up again from day +79 until today. Total lymphocyte count did not change. NK cells and plasmablasts were practically removed from peripheral blood since the first infusion of daratumumab until day +79. aPL are expressed in U/mL. Total serum Immunoglobulins are expressed in g/L and D-dimer in mg/dL. Lymphocyte subpopulations are expressed as % of total lymphocytes or in counts/mL.*

daratumumab infusions, the patient experienced a remarkable improvement in dyspnea, pleuritic right pain, and the swelling of a leg, so that she could be discharged on day +7 after the start of anti-CD38 therapy. Although we saw an impressive change on the patient's symptoms right after the first daratumumab infusion, we could not overlook the effect of the combined measures on this initial good outcome.

However, we believe that the beneficial effects observed for two years, with absence of the continuous thrombotic episodes that the patient suffered before the intervention, could be explained by the decrease in aPL titers. Despite we did not achieve a total negativization of aPL, the clinical improvement might be explained by the random depletion of the plasma cell clones producing the more pathogenic antibodies.

On the follow-up, the patient acknowledged incomplete adherence to the treatment with missing of some doses of anticoagulant therapy at multiple time points. One of them led to a new VT two years later, coinciding with the increase in aPL levels, which reached or surpassed baseline levels. This behavior is understandable as we did not target circulating naïve and memory B cells, which would be responsible for the long-term repopulation of aPL. In a recent review on emerging B-cell therapies in SLE by Ayse Bag-Ozbek and Joyce S Hui-Yuen (48), they commented on further treatments employed in two patients with SLE who received four weekly doses of 16 mg/kg daratumumab as part of a clinical trial (44). These two patients were treated with belimumab starting four weeks after the end of therapy with daratumumab to prevent the activation and proliferation of autoreactive B cells.

CONCLUSION

The treatment with daratumumab showed an adequate safety profile, was well tolerated, and was associated with clinical improvement, although levels of aPL only showed a partial response. Based on our experience and on data now available in the literature, we suggest that anti-CD38 therapy could be a valuable tool to consider for APS patients who are refractory to the anticoagulant therapy, alone or in combination with B-cell depleting mAbs. Further investigation is needed to better elucidate the role and optimal timing and doses of daratumumab in the treatment of refractory APS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Hospital Universitario 12 de Octubre, Avenida

de Córdoba SN. Madrid, SPAIN. The patient provided her written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DP and RD-S equally contributed in patient evaluation, therapy administration, follow-up and preparation of this case report. DP and AS designed the strategy of therapy. OC-M, AL, EP-A, CL and AS wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Antiphospholipid Antibodies and Infection: *Non Nova Sed Nove*

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The clinical significance of antiphospholipid antibodies (aPL) in the context of infections has attracted attention since their first discovery in patients with syphilis. In fact, the recognition of aPL in patients with infections has been described in parallel to the understating of the syndrome. Since the first description of aPL-positive tests in three patients with COVID-19 diagnosed in January 2020 in Wuhan, China, a large number of studies took part in the ongoing debate on SARS-2-Cov 2 induced coagulopathy, and many following reports speculated a potential role for aPL. In order to get further insights on the effective role of detectable aPL in the pro-thrombotic status observed in COVID-19 patients, we performed an observational age-sex controlled study to compare the aPL profile of hospitalized patients with COVID with those observed in a) patients with thrombotic APS and b) patients with cultural/serologically-proved infections. Our data showed positive aPL testing in about half of the patients (53%) with COVID-19 and patients with other viral/bacterial infections (49%). However, aPL profile was different when comparing patients with overt APS and patients with aPL detected in the contest of infections. Caution is therefore required in the interpretation and generalization of the role of aPL s in the management of patients with COVID-19. Before introducing aPL testing as a part of the routine testing in patients with COVID-19, larger well-designed clinical studies are required. While the pro-thrombotic status in patients with COVID-19 is now unquestionable, different mechanisms other than aPL should be further investigated.

Keywords: antiphospholipid antibodies, infection, COVID-19, antiphospholipid antibody syndrome, thrombosis

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INTRODUCTION

The antiphospholipid syndrome (APS) is a systemic autoimmune condition characterized by the persistent elevation of antiphospholipid antibodies (aPL), such as anticardiolipin antibodies (aCL), lupus anticoagulant (LA), and anti-beta2 Glycoprotein 1 (aβ2GPI), in patients with thromboembolic events and/or pregnancy-related morbidity (1) (**Box 1**). The clinical significance

BOX 1 | Classification criteria of the Antiphospholipid Syndrome.

Clinical criteria

- Thrombosis affecting the arteries, veins, or small blood vessels and/or
- Adverse outcomes during pregnancies (three or more spontaneous abortions before 10th week of pregnancy, unexplained fetal deaths at or beyond 10th week of pregnancy, or premature births before 34th week of pregnancy due to severe preeclampsia or eclampsia)

Laboratory criteria (antiphospholipid antibody tests, to be confirmed at 12 weeks)

- Positive lupus anticoagulant test and/or
- Positive anticardiolipin antibody (aCL) IgG and/or IgM and/or
- Positive anti-Beta-2-glycoprotein-I antibody (aβ2GPI) IgG and/or IgM

of aPL in the context of infections has attracted attention since their first discovery in patients with syphilis (2). In fact, the recognition of aPL in patients with infections has been described in parallel to the understating of the syndrome (3). Since the global outbreak of the COVID-19 pandemic, a potential relationship between the presence of aPL and the new has been largely debated.

First, Zhang and co-workers (4) reported a 69-year-old man with COVID-19 diagnosed in January 2020 in Wuhan, China, along with two other critically ill patients with COVID-19 who were also seen in the same intensive care unit. aPL were detected in all three patients. Since then, a large number of studies took part in the ongoing debate on SARS-2-Cov 2–induced coagulopathy, and many following reports speculated a potential role for aPL.

Recently, Zou et al. (5), when testing 172 patients hospitalized with COVID-19 for an extended panel of aPL, found that aPL were present in up to 52% of serum samples using the manufacturer's threshold and in 30% using a more stringent cut-off (≥40 ELISA-specific units). In detail, among the various aPL antibodies tested, anti-phosphatidylserine/prothrombin (aPS/PT) IgG had the highest prevalence (24%), followed by aCL IgM (23%) and aPS/PT IgM (18%). Forty-one patients (24%) were positive for more than one type of aPL antibody, and 13 (8%) were positive for more than two types of aPL antibody. Fifty-two patients (30%) had at least one moderate- to high-titer aPL antibody. Interestingly, they reported that IgG fractions isolated from patients with COVID-19 and high serum titers for aPS/PT IgG increased thrombus extension in a murine model. While the pro-thrombotic profile of patients with COVID-19 is unquestionable, the specific role of aPL in this setting still requires further considerations.

Do the detectable aPL actively participate in the pro-thrombotic status observed in COVID-19 patients or they represent an epiphenomenon in the context of the infection, as previously described in other settings?

In order to get further insights on the effective role of detectable aPL in the pro-thrombotic status observed in COVID-19 patients, we performed an observational age-sex controlled study to compare the aPL profile of hospitalized patients with COVID with those observed in a) patients with thrombotic APS and b) patients with cultural/serologically-proved infections.

METHODS

We included 261 patients (divided in three age and sex-matched controls groups of 87 patients):

1) Consecutive PCR-confirmed COVID-19–infected patients admitted at the AO Ordine Mauriziano Hospital, Torino, Italy
2) Age- and sex-matched controls with viral and bacterial infections* and no previous history of thrombotic events attending the S. Giovanni Bosco Hospital, Torino, Italy
3) Age and sex-matched patients with APS fulfilling Sidney's criteria (1) admitted at the S. Giovanni Bosco Hospital, Torino, Italy

The aetiology of infections was: 24 cases of Treponema pallidum, 18 cases of cytomegalovirus, 10 cases of Influenza H3N2, 10 of E. coli, 8 of Streptococcus pneumoniae, 7 of parvovirus B19, 7 of Epstein-Barr virus, and 2 of Pseudomonas aeruginosa (**Table 1**).

The IgG/IgM isotype for aCL, aβ2GPI and aPS/PT were detected by commercial ELISA (Inova Diagnostics, Inc., San Diego, CA, US).

LA was tested as per the current criteria from the International Society of Thrombosis and Haemostasis (ISTH) Subcommittee on LA-Phospholipid-dependent antibodies (6).

The significance of baseline differences was determined by the chi-squared test, Fisher's exact test or the unpaired t-test, as appropriate. A two-sided P-value <0.05 was statistically significant. All statistical analyses were performed using SPSS version 26.0 (IBM, Armonk, NY, USA).

RESULTS

A total of 261 patients were enrolled in this study, 87 patients for each group: patients with COVID-19 infection, matched-controls with infections other than COVID-19 and matched-APS patients. As per classification criteria, all patients with APS were positive for aPL, while a high rate of patients positive for at least one aPL (IgG/IgM) was observed similarly in patients with COVID-19 infection (46; 52.9%) and controls suffering with other infections (43; 49.4%).

PCR-confirmed COVID-19–infected patients were tested for aPL at the time of the admission in the Hospital, 84 patients (96.7%) were treated with low molecular weight heparins. Only one patient developed a deep vein thrombosis during the admission. **Table 1** resumes the rate of positive antiphospholipid antibodies' testing and relative differences between groups.

When focusing on criteria aPL, as expected, APS patients had significantly higher rates of positive testing for LA when compared with the other groups [87.4% *vs.* 29.9% (COVID-19) and *vs.* 47.1% (infections)] and for IgG isotype of aß2GPI [47.1% vs. 13.8% (COVID-19) and *vs.* 12.6% (infections)] and aCL [48.3% *vs.* 12.6% (COVID-19) and *vs.* 12.6% (infections)]. Also, when looking at multiple criteria aPL positive test (considering IgG and/or IgM isotypes), the APS group had significantly higher rates of double aPL positive testing [43.7% *vs.* 8% (COVID-19) and *vs.* 18.4%

TABLE 1 | Rate of positive antiphospholipid antibodies' testing and relative differences between groups.

	Group A Patients with infections (n = 87)	P value (Group A *vs*. B)	Group B COVID-19 Patients (n = 87)	P value (Group B *vs*. C)	Group C Patients with APS (n = 87)	P value (Group B *vs*. C)
Lupus Anticoagulant positive (n)	41	0.02	26	<0.0001	76	<0.0001
β2GPI IgM positive(n)	17	0.002	4	n.s.	9	0.046
β2GPI IgM titers (U/mL; mean ± SD)	25.8 ± 57.1	0.0003	2.7 ± 5.6	n.s.	9.9 ± 28.3	n.s.
β2GPI IgG positive(n)	11	n.s.	12	<0.0001	41	<0.0001
β2GPI IgG titers (U/mL; mean ± SD)	12 ± 14.6	n.s.	8 ± 8.9	<0.0001	57.9 ± 92.4	<0.0001
aCL IgM positive(n)	18	0.0004	4	<0.0001	28	0.0015
aCL IgM titers (U/mL; mean ± SD)	19.8 ± 33	n.s.	3.8 ± 5.3	n.s.	17.5 ± 36.8	n.s.
aCL IgG positive(n)	11	n.s.	11	0.002	42	0.002
aCL IgG titers (U/mL; mean ± SD)	14.7 ± 27.8	n.s.	10.1 ± 16.3	<0.0001	69.3 ± 108.3	<0.0001
aPS/PT IgM positive (n)	22	0.0005	7	<0.0001	51	<0.0001
aPS/PT IgM titers (U/mL; mean ± SD)	36.1 ± 48.9	<0.0001	10.6 ± 15.7	<0.0001	137 ± 133.5	<0.0001
aPS/PT IgG positive (n)	0	n.s.	0	<0.0001	34	<0.0001
aPS/PT IgG titers (U/mL; mean ± SD)	9.3 ± 6.2	<0.0001	3.7 ± 4.3	<0.0001	83.8 ± 101.9	<0.0001
Double Criteria aPL positive (IgG/IgM) (n)	16	0.043	7	<0.0001	38	<0.0001
Triple Criteria aPL positive (IgG/IgM) (n)	7	n.s.	2	<0.0001	35	<0.0001
Triple Criteria aPL (IgG/IgM) and aPS/PT (IgG/IgM) positive (n)	3	n.s.	0	<0.0001	30	<0.0001

APS, antiphospholipid Syndrome; aPL, antiphospholipid antibodies; aPS/PT, anti-phosphatidylserine/prothrombin antibodies; aβ2GPI, anti-β2-glycoprotein-I antibodies; aCL, anticardiolipin antibodies; Ig, immunoglobulin; n.s., non significant.

(infections)], triple aPL positive testing [40.2% *vs*. 2.2% (COVID-19) and *vs*. 8% (infections)].

The trend was confirmed also when considering aPS/PT testing, as non-criteria aPL. APS patients were the only group with patients that tested positive for aPS/PT IgG (39.1%) and had significantly higher rates of aPS/PT IgM testing [58.6% *vs*. 8% (COVID-19) and *vs*. 25.3% (infections)]. Additionally, 34.5% of APS patients tested positive for both all three criteria aPL and aPS/PT IgG/M (tetra positive patients), when compared to only three patients of the infections group and no patients in the COVID-19 infection group.

The results of the analysis, for both criteria and non-criteria aPL, were also confirmed when analyzing the titers of the aPL tested (**Table 1**).

Figure 1 graphically illustrates the different rate of aPL positive testing between groups.

No patients with aPL-positive test reported any thrombotic events (both COVID-19 and infections group). In the COVID-19 group, we observed that no previously positive solid phase aPL test was confirmed when re-testing 12 patients at more than 12 weeks part. Of those, of the five LA-positive tests, only two were confirmed, albeit with a marked reduction of LA potency.

DISCUSSION

Our data showed positive aPL testing in about half of the patients (53%) with COVID-19 and patients with other viral/bacterial infections (49%). In detail, we found that a positive test for LA can be detected in up to one out of three symptomatic COVID-19 patients when tested according to the ISTH (6). However, the so called triple aPL positivity (concomitant presence of LA, aCL, and aβ2GPI antibodies), the *aPL* profile most strongly associated with a thrombotic event in patients with APS, has been observed in only two patients (2%). More importantly, most of the aCL and aβ2GPI antibodies positive testing was detected at the low-medium titer. Interestingly, in our cohort, no patient with infection (both in the COVID-19 or infection groups) was found positive for aPS/PT IgG.

aPL positivity are known to be detectable during infections, to include viral diseases, such as HIV and hepatitis C. The presence of aPL in these contexts is often transient and almost always non-specific (non-thrombosis-related). While syphilis is notorious for the possible determination of aPL, when considering our cohort, we did not observe a different rate of aPL positive testing between patients with syphilis when compared to other infections. However, it is likely that the sample size limited the feasibility of any additional sub-analysis.

When comparing our data to Zuo et al. (5), some considerations are worth noting. So far, LA-positive testing was the mostly frequently reported among patients with COVID tested for aPL. From this perspective, as a limit to their analysis, they acknowledged that the LA test was not performed given lack of access to fresh plasma samples. When considering solid-phase aPL assays, methods that are in principle insensitive to anticoagulation and other confounding agents, the presence of aPL in patients with COVID-19 was recently reported in a handful of case reports and small cohorts of patients (7, 8). While encouraging, these data are limited and its interpretation remains controversial, with some investigators proposing an important role of aPL in COVID-19 patients (7, 8) while others suggesting no association between aPL and thrombotic events (9, 10). There is no information on the antigen specificity of COVID-19 aPL in comparison with APS antibodies. Such information and a larger study, possibly multicenter, may be instrumental to clarify the real clinical value of these autoantibodies.

Our results support what Borghi et al. suggested (9). In fact, when testing for aPL by both ELISA and chemiluminescence 122 sera of patients with COVID, they (9) found that anti-β2GPI IgG

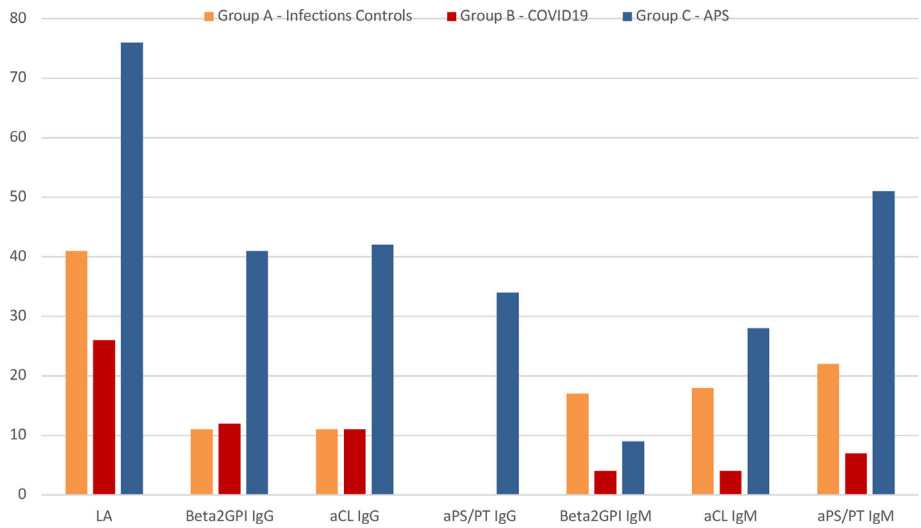

FIGURE 1 | Graphical representation of the rate of antiphospholipid antibodies positive patients between groups. APS, antiphospholipid syndrome; LA, lupus anticoagulant; aPS/PT, anti-phosphatidylserine/prothrombin antibodies; aβ2GPI, anti-β2-glycoprotein-I antibodies; aCL, anticardiolipin antibodies; Ig, immunoglobulin.

were detectable in about 15%, with IgA and IgM respectively in 6.6 and 9.0% of patients. aCL IgG/IgM was detected in 5.7/6.6% and aPS/PT IgG/IgM were detectable in 2.5% and 9.8%. Critically, no association between thrombosis and aPL was found. Reactivity against domain 1 and 4 to 5 of β2GPI was limited to 3/58 (5.2%) tested sera for each domain and did not correlate with aCL/anti-β2GPI nor with thrombosis.

Some limitations should be acknowledged. Patients enrolled in the control infectious group were chart review-selected by matching cases for sex and age group with 1 year difference allowance. Taking this into account, it was out of the scope of this paper to report on any estimations on specific infection rates or epidemiological inference for this group and to provide evidence on the potential impact of clinical disease on aPL development. Similarly, it was out of the scope of this study to investigate thrombosis rates in patients with COVID-19 infections.

In such a dramatic moment globally, researchers are called to the urgent need for a better understanding of COVID-19–related syndrome. On the other hand, before solid evidence is available, caution is warranted to minimize the risk of unjustified requests for tests to be performed in laboratories that are already overloaded during the ongoing health emergency. Besides, with the current level of evidence, the new detection of aPL in a patient with COVID-19 should not guide the management of the anti-thrombotic therapy that should be based on the available international guidelines.

In conclusion, caution is therefore required in the interpretation and generalization of the role of aPL such as aPS/PT in the management of patients with COVID-19. Before introducing aPL testing as a part of the routine testing in patients with COVID-19, larger well-designed clinical studies are required. While the pro-thrombotic status in patients with COVID-19 is now unquestionable, different mechanisms other than aPL should be further investigated.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Interaziendale Città Di Torino. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SS and MB designed the study, analyzed the data, and drafted the manuscript. MR, MB, DC, CN, MTB, RC, BB, SG, and DR participated in case collection, laboratory analysis, and data analysis and criticially participated in the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comparison of Different Test Systems for the Detection of Antiphospholipid Antibodies in a Chinese Cohort

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Background: Diagnosis of antiphospholipid syndrome (APS) is based on the positivity of laboratory criteria antiphospholipid antibodies (aPLs). Test results for aPLs could be contradictory among different detection methods as well as commercial manufacturers. This study aimed to assess and compare the diagnostic and analytic performances of four commercial assays prevalently used in China.

Methods: A total of 313 patients including 100 patients diagnosed with primary APS, 52 with APS secondary to SLE, 71 with SLE, and 90 health controls were recruited. Serum IgG, IgM, and IgA for aCL, and aβ2GPI antibodies were detected with two ELISA and two CLIA systems, and test system with the best diagnostic value was explored of its correlation with key clinical features.

Results: CLIA by YHLO Biotech Co. was considered as the system with the best predictive power, where 58.55 and 57.89% of APS patients were positive for aCL or aβ2GPI for at least one antibody (IgG or IgM or IgA). Overall, CLIA showed better performance characteristics than traditional ELISA test systems.

Conclusion: CLIA was considered as a better platform for aPL detection in APS diagnosis. A combination of other detection platforms could assist in differential diagnosis as well as in identifying high-risk patients.

Keywords: antiphospholipid antibodies, antiphospholipid syndrome, chemiluminescent immunoassay, enzyme-linked immunosorbent assay, anti-β2 glycoprotein-I, anti-cardiolipin

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INTRODUCTION

The antiphospholipid syndrome (APS) is defined by the development of venous/arterial thromboses or by the occurrence of obstetrical events including recurrent fetal losses or increased perinatal morbidity, with the persistent presence of antiphospholipid antibodies (aPLs). According to the 2006 APS classification criteria, APS diagnosis is based on the positivity of at least one of the clinical criteria as well as one of laboratory criteria including lupus anticoagulant (LA), high level of anti-cardiolipin (aCL), anti-β2 glycoprotein-I (aβ2GPI) immunoglobulin isotype G (IgG) or M (IgM) (1). More recently, non-criteria aPLs including anti-aCL or anti-b2GPI IgA, anti-phosphatidylserine–prothrombin (aPS/PT) complex, anti-annexin A5 antibodies (aAnxV), *etc.* are receiving increasing attention (2).

APS could be associated with several severe clinical outcomes such as pulmonary embolism, acute myocardial infarction, and stroke, which demand immediate appropriate intervention. On the other hand, anticoagulant treatment commonly utilized for APS could increase bleeding risk for susceptible patients. Since aPL detection comprise a large part of APS diagnosis, a detection system with high sensitivity and specificity is required in order to timely identify APS patients as well as provide accurate clinical intervention (3). Besides, evaluation of aPLs could also contribute to prognosis and risk assessment for associated clinical manifestations (4, 5).

Numerous guidelines and studies concerning aCL and aβ2GPI tests have been published (6). However, test results for aPLs remain contradictory among different detection methods as well as commercial manufacturers, probably due to the lack of standardization for cut-off values, method of calibration and quantitation, choice of solid phase and coating, type and source of antigen, and other analytic problems (7–9). Traditionally, enzyme-linked immunosorbent assay (ELISA) was applied due to its relative time and cost-efficiency. In recent years, novel automating detection systems, such as chemiluminescent immunoassay (CLIA), addressable laser bead immunoassay (ALBIA), line immunoassay (LIA), *etc.* have been introduced for aPL detection, and promising results have been yielded (10–14). Automatization can improve the reproducibility and reduce interlaboratory variation, yet may show distinct performance characteristics compared to ELISA (15, 16).

More specifically, in China, home-conducted ELISA is still most widely applied at laboratories for APS diagnosis. However, an increasing number of automated analyzers have been equipped by large general hospitals with high application potentials. Regarding commercially available systems, most studies focused on measuring and comparing only one assay to laboratory-conducted ELISA (17). However, little attention has been paid to simultaneously evaluate different test systems that are commonly chosen. The aim of this study was to assess and compare the diagnostic and analytic performances of four commercial assays prevalently used in China, including two ELISA and two CLIA systems, in a Chinese prospective APS cohort. Detection of IgG, IgM, and IgA for aCL and aβ2GPI antibodies was evaluated, and a test system with the best diagnostic value was explored of its correlation with key clinical features.

PATIENTS AND METHODS

Patients Recruitment

This was a single-center, prospective cohort study conducted at Peking Union Medical College Hospital (PUMCH) and the National Clinical Research Center for Dermatologic and Immunologic Diseases (NCRC-DID) from May 2017 to January 2020. A total of 313 consecutive patients were included in this study, of which 100 patients had been diagnosed with primary APS (PAPS group), 52 with APS secondary to SLE (SAPS group), 71 with SLE (SLE group), and 90 healthy controls (HC group). Diagnosis of APS was defined by clinicians according to the 2006 Sydney revised classification criteria (1). According to the criteria, IgG and IgM aCL and a2GPI were analyzed with standardized ELISA (INOVA Diagnostics) at the Key Laboratory. Lupus anticoagulant was detected and evaluated according to the ISTH recommendations. Dilute Russell viper venom time (dRVVT) testing and activated partial thromboplastin time were measured, where LAC was considered positive if the ratio of screen/confirm time ratio was >1.20. Diagnosis of SLE was based on the 1997 ACR criteria and confirmed by the 2019 EULAR/ACR criteria. Clinical manifestations were recorded for PAPS, SAPS, and SLE groups, including vascular thrombosis (arterial or venous), pregnancy morbidity, and extra-criteria manifestations, including thrombocytopenia, heart valve disease, autoimmune hemolytic anemia, and neurological disorders, *etc.* For the HC group, only aPL serology information was present. For each subject, 4 ml of blood was collected with the help of a BD vacutainer without anticoagulants. Blood samples were allowed to clot at room temperature for 1 h and then centrifuged at 4°C for 5 min at 3,000 rpm. Serum was collected and stored at −80°C. No sample was exposed to more than one freeze–thaw cycle before analysis. The study was approved by the ethics committee at PUMCH and fulfilled the ethical guidelines of the declaration of Helsinki. All subjects gave written informed consent.

Laboratory Tests

For each study subject, IgG, IgM, and IgA isotypes of aCL and aβ2GPI were analyzed with four systems listed below: a. iFlash CLIA kits provided by YHLO Biotech Co., Shenzhen, China (Y-CLIA); b. QUANTA Flash® CLIA kits provided by INOVA Diagnostics, Inc., San Diego, CA, US, Werfen Group as sales agent (W-CLIA); c. QUANTA Lite™ ELISA kits provided by INOVA Diagnostics, Inc., San Diego, CA, US, Werfen Group as sales agent (W-ELISA); d. AESKULISA® ELISA test kits provided by Aesku.Diagnostics GmbH & Co. KG, Wendelsheim, Germany (A-ELISA). Detailed characteristics of test systems from different manufacturers were summarized in **Table 2**. Cut-off values were defined for each system as recommended by the manufacturer.

Statistical Analysis

Statistical analysis was performed using SPSS 26.0 or R (version 3.6.2). The χ^2 test or Fisher's exact test was used for comparison of categorical variables, and Wilcoxon test was used for continuous variables after normality was explored with the

Shapiro–Wilk test. Sensitivities, specificities, and accuracies in APS diagnosis were compared in the McNemar test. Youden Index, positive and negative predictive values (PPV and NPV), and odds ratio (OR) with 95% confidence interval (95% CI) were also shown. Correlation of different aPL isotype levels with clinical manifestations was calculated, and clinical events with 95% CI were displayed. Two-tailed values of p less than 0.05 were considered statistically significant.

RESULTS

Patient Characteristics

Among 152 APS patients, there were 63 (63.0%) females for PAPS, 46 (88.5%) for SAPS, and the mean age for each was 36.3 and 32.9 years (**Table 1**). Mean age was 30.1+/−8.2 years in the SLE group, of which 61 (85.9%) were female, while the HC group had 41 (45.6%) female and a mean age of 43.4+/−12.2. Detailed clinical manifestations were recorded for both APS and SLE patients and were shown. Thrombosis was most commonly present, with 80 (80.0%) for PAPS and 39 (75%) for SAPS, but not in the SLE group. Patients with history of arterial or venous thrombosis were recorded for APS patients. Pregnancy morbidity, history of adverse pregnancy, microangiopathy, and LA were also observed in both PAPS and SAPS group. Of all the clinical manifestations, the prevalence of thrombocytopenia was significantly different between PAPS and SAPS group (χ^2 = 4.382, p = 0.036).

Assay Characteristics

As summarized in **Table 2**, the coating, conjugation, calibration, and cut-off values with their calculation were listed for four commercial test systems. More specifically, Y-CLIA conducted paramagnetic particle chemiluminescent immunoassay using a fully automated iFlash 3000 Chemiluminescence Immunoassay Analyzer. Recommended values with best sensitivity, specificity, and false positive results of healthy donors against APS, SLE, and other autoimmune disease patients were chosen for all antibody isotypes. For W-CLIA, antigen-specific paramagnetic bead chemiluminescent immunoassay was conducted employing the fully automated BIO-FLASH CLIA instrument. Cut-off values for all antibodies were calculated using the 99th percentile in healthy groups. W-ELISA was a semi-quantitative enzyme linked immunosorbent assay manually conducted according to the manufacturer's instruction.

TABLE 1 | Demographic and clinical variables of subjects (n = 313).

	APS (152)		SLE (71)	Health controls (90)
	Primary (100)	Secondary (52)		
Gender (female/male)	63/37	46/6	61/10	41/49
Mean age (years ± SD)	36.3 ± 12.1	32.9 ± 10.2	30.1 ± 8.2	43.4 ± 12.2
Clinical manifestations				
Thrombosis, n (%)	80 (80.0%)	39 (75.0%)	0	NA
Pregnancy morbidity, n (%)	33 (33.0%)	17 (32.7%)	0	NA
Thrombosis + pregnancy morbidity, n (%)	13 (13.0%)	4 (7.7%)	0	NA
LA, n (%)	73 (73.0%)	44 (84.6%)	17 (23.9%)	NA
History of arterial thrombosis, n (%)	43 (43.0%)	21 (40.4%)	0	NA
Stroke, n (%)	4 (4.0%)	2 (3.8%)	0	NA
Coronary heart disease, n (%)	9 (9.0%)	2 (3.8%)	0	NA
Eye involvement, n (%)	3 (3.0%)	1 (1.9%)		
Lower limb artery occlusion, n (%)	1 (1.0%)	0	0	NA
History of venous thrombosis, n (%)	47 (47.0%)	24 (46.2%)	0	NA
Deep vein thrombosis, n (%)	19 (19.0%)	7 (13.5%)	0	NA
Pulmonary embolism, n (%)	19 (19.0%)	2 (3.8%)	0	NA
Upper limb vein thrombosis, n (%)	0	1 (1.9%)	0	NA
Renal vein thrombosis, n (%)	1 (1.0%)	0	0	NA
Portal vein thrombosis, n (%)	4 (4.0%)	1 (1.9%)	0	NA
Cerebral venous and sinus thrombosis, n (%)	3 (3.0%)	1 (1.9%)	0	NA
Central retinal venous occlusion, n (%)	1 (1.0%)	0	0	NA
Microangiopathy, n (%)	57 (57.0%)	24 (46.2%)	0	NA
Thrombocytopenia, n (%)	38 (38.0%)	*29 (55.8%)	21 (29.6%)	NA
Heat valve disease, n (%)	0	6 (11.5%)	0	NA
Non-stroke CNS manifestations, n (%)	4 (4.0%)	4 (7.7%)	0	NA
Antiphospholipid syndrome nephropathy, n (%)	6 (6.0%)	2 (3.8%)	0	NA
Autoimmune hemolytic anemia, n (%)	1 (1.0%)	5 (9.6%)	0	NA
Thrombotic Microangiopathy, n (%)	0	1 (1.9%)	0	NA
Hemolytic uremic syndrome, n (%)	1 (1.0%)	0	0	NA
History of adverse pregnancy, n (%)	37 (37.0%)	21 (40.4%)	4 (5.6%)	NA
Early fetal loss (<10 weeks), n (%)	12 (12.0%)	8 (15.4%)	4 (5.6%)	NA
Late fetal loss (10–28 weeks), n (%)	19 (19.0%)	12 (23.1%)	0	NA
Placenta insufficiency, n (%)	14 (14.0%)	7 (13.5%)	0	NA

*p = 0.036, significant different from primary APS.
NA, Not Available.

TABLE 2 | Characteristics of test systems from different manufacturers.

	Assay	Coating well/ particle	Conjugate	Calibration	Manufacturer's cutoff	Calculation
iFlash (Y-CLIA)	Anti-cardiolipin IgM, IgG, IgA	Human cardiolipin	Acridine anti-human IgM/IgG/ IgA antibody	Internal standard: Louisville APL Diagnostics	10 U/ml	59/38 APS and other AID patients, 241/262 blood bank donors, a recommended value
	Anti-β2 glycoprotein I IgM, IgG, IgA	Human β2 glycoprotein I	Acridine anti-human IgM/IgG/ IgA antibody	Internal standard: Louisville APL Diagnostics	20 U/ml	62/72 APS and other AID patients, 238/308 blood bank donors, a recommended value
QUANTA Flash (W-CLIA)	QUANTA Flash aCL IgG, IgM, IgA	Bovine cardiolipin with human β2GPI	Isoluminol anti-human IgM/IgG/ IgA antibody	Internal standard: HCAL for IgG and EY2C9 for IgM	20 CU	250/262 blood bank donors, 99th percentile
	QUANTA Flash β2GP1 IgG, IgM, IgA	Human β2GPI	Isoluminol anti-human IgM/IgG/ IgA antibody	Internal standard: HCAL for IgG and IgA, EY2C9 for IgM	20 CU	250–252 blood bank donors, 99th percentile
QUANTA Lite (W-ELISA)	QUANTA Lite ACA IgG III, IgM III, IgAIII	Purified cardiolipin and bovine β2GPI	HRP goat anti-human IgM/IgG/ IgA antibody	Internal standard: HCAL for IgG and EY2C9 for IgM	20 MPL, GPL, APL	488–489 normal donors, a recommended value
	QUANTA Lite β2 GPI IgG, IgM, IgA	Purified β2GPI	HRP goat anti-human IgM/IgG/ IgA antibody	Internal standard: human serum antibodies to β2GPI	20 SMU, SGU, SAU	11–313 normal donors, a recommended value
AESKULISA (A-ELISA)	AESKULISA Cardiolipin-GM, Cardiolipin-A	Purified cardiolipin and bovine β2GPI	HRP anti-human IgM/IgG/IgA antibody	Internal standard: HCAL for IgG and EY2C9 for IgM, Louisville APL for IgA	18 MPL, GPL, APL	NA
	AESKULISA β2-Glyco-GM, β2-Glyco-A	Purified β2GPI	HRP anti-human IgM/IgG/IgA antibody	Internal standard: HCAL for IgG and EY2C9 for IgM	18 U/ml	NA

MPL, GPL, and APL for IgM, IgG, and IgA phospholipid units; SMU, SGU, SAU for standard IgM, IgG, and IgA units, HRP for horseradish peroxidase. NA, Not Available.

Cut-off values were set based on the evaluation of normal and positive antibody samples. For A-ELISA, assay was also manually conducted following manufacturer's protocols, yet no information was provided for cut-off value calculation.

Predictive Power of aPLs for Different Test Systems

Antibody results obtained from four test systems were evaluated for diagnostic power with sensitivity, specificity, accuracy, Youden Index, PPV, and NPV in APS diagnosis from the HC group in **Table 3**. For each antibody type, sensitivity, specificity, and accuracy were compared first between the same test methods (*i.e.*, Y-CLIA against W-CLIA, W-ELISA against A-ELISA). The better system from each method, if identified, was then compared to determine the best system, which was further evaluated for clinical manifestation prediction. As shown in **Table 3**, the accuracy of aCL IgG was significantly higher for Y-CLIA than W-CLIA ($p < 0.001$), and A-ELISA than W-ELISA ($p = 0.035$). The sensitivity ($p < 0.001$) and accuracy ($p < 0.001$) were both significantly higher for Y-CLIA method. For aCL IgM, sensitivity and accuracy were significantly higher for W-ELISA than A-ELISA ($p < 0.001$). As for aCL IgA, Y-CLIA and A-ELISA were selected respectively for comparison, and the specificity of the former was significantly higher ($p = 0.031$). Sensitivity and accuracy of positivity of aCL IgG, IgM, or IgA were also significantly higher for Y-CLIA than for W-CLIA ($p < 0.001$). Y-CLIA and W-ELISA were selected as better systems for positivity of aCL IgG or IgM, and significant difference was observed for accuracy ($p = 0.022$). Concerning aβ2GPI, Y-CLIA and W-CLIA were selected for comparison of IgM, whose specificity ($p = 0.049$) was higher that the former. Sensitivity and accuracy of positivity of aβ2GPI IgG, IgM, or IgA, as well as those of aCL IgG or IgM, were all significantly higher for Y-CLIA. All in all, Y-CLIA was considered as a system with the best predictive power.

Similarly, the sensitivity, specificity, and accuracy were also compared among four systems in identifying thrombosis and pregnancy morbidity (**Table 4**). For thrombosis events, significant results for sensitivity and accuracy of aCL and aβ2GPI positivity were all higher for Y-CLIA than for W-CLIA and for W-ELISA than for A-ELISA. Y-CLIA still showed higher accuracy ($p = 0.022$ for aCL IgG or IgM and $p = 0.001$ for aβ2GPI IgM). As for pregnancy morbidity, significant results for specificity and accuracy of aCL and aβ2GPI positivity were significantly higher for W-CLIA than for Y-CLIA and for A-ELISA than for W-ELISA.

Distribution of aPL Test Results

As different cut-off values were used by four test systems, the distribution of aPL test results from different manufacturers among patient groups were calculated with lg[(test result/cutoff value) +1] so that they could be visualized together as positive

TABLE 3 | Comparison of the predictive power of aPL tests from different test systems in APS diagnosis.

			Sensitivity (%)	Specificity (%)	Accuracy (%)	Youden Index	PPV (%)	NPV (%)
aCL IgG	CLIA	#Y*	50.66	100.00	69.01	0.507	100.00	54.55
		W	40.13	95.45	60.42	0.356	93.85	48.00
	p_C		1.000	0.134	**<0.001**			
	ELISA	W	37.50	95.12	57.69	0.326	93.44	45.09
		A*	37.09	100.00	60.58	0.371	100	48.65
	p_E		1.000	1.000	**0.035**			
	$p_{C/E}$		**<0.001**	NA	**<0.001**			
aCL IgM	CLIA	Y	16.45	96.67	46.28	0.131	89.29	40.65
		W	13.82	100.00	44.95	0.138	100.00	39.63
	p_C		0.344	0.250	0.332			
	ELISA	W*	33.55	98.78	56.41	0.324	98.08	44.51
		A	8.61	97.78	41.90	0.064	86.67	38.94
	p_E		**<0.001**	1.000	**<0.001**			
	$p_{C/E}$							
aCL IgA	CLIA	#Y*	23.03	98.89	51.24	0.219	97.22	43.20
		W	13.82	100.00	44.49	0.138	100.00	39.07
	p_C		**0.001**	1.000	**<0.001**			
	ELISA	W	4.61	98.78	37.61	0.034	87.50	35.84
		A*	30.46	92.22	53.5	0.227	86.79	44.15
	p_E		**<0.001**	0.063	**<0.001**			
	$p_{C/E}$		0.071	**0.031**	0.511			
aCL IgG or IgM or IgA	CLIA	Y*	58.55	95.56	72.32	0.542	95.70	57.72
		W	46.71	95.24	64.08	0.419	94.67	49.69
	p_C		**<0.001**	1.000	**<0.001**			
	ELISA	W	53.95	93.75	67.77	0.477	94.25	51.72
		A	51.66	91.11	66.39	0.428	90.70	52.90
	p_E		0.608	1.000	0.775			
	$p_{C/E}$							
aCL IgG or IgM	CLIA	#Y*	58.55	96.67	72.73	0.553	96.74	58.00
		W	46.05	95.29	63.72	0.414	94.59	49.69
	p_C		**<0.001**	0.625	**<0.001**			
	ELISA	W*	53.95	95.00	68.10	0.489	95.35	52.05
		A	41.06	97.78	62.24	0.389	96.88	49.72
	p_E		**0.001**	0.375	0.302			
	$p_{C/E}$		0.349	0.687	**0.022**			
aβ2GPI IgG	CLIA	Y	46.71	100.00	66.53	0.467	100.00	52.63
		W	50.00	97.67	67.22	0.477	97.44	52.50
	p_C		0.442	0.500	1.000			
	ELISA	W*	31.58	95.51	55.19	0.271	92.31	44.97
		A	23.18	100.00	51.86	0.232	100.00	43.69
	p_E		**0.004**	0.125	0.152			
	$p_{C/E}$							
aβ2GPI IgM	CLIA	#Y*	21.1	97.78	49.58	0.189	94.12	42.31
		W	9.21	100.00	42.26	0.092	100.00	38.67
	p_C		**<0.001**	0.500	**<0.001**			
	ELISA	W*	15.13	100.00	46.02	0.151	100.00	40.28
		A	7.95	98.89	41.91	0.068	92.31	39.04
	p_E		**0.021**	1.000	0.064			
	$p_{C/E}$		**0.049**	NA	0.136			
aβ2GPI IgA	CLIA	Y	16.45	98.89	47.11	0.153	96.15	41.20
		W	13.82	100.00	44.95	0.138	100.00	39.63
	p_C		0.344	1.000	0.118			
	ELISA	W*	11.84	96.51	42.43	0.083	85.71	38.25
		A	6.62	98.89	41.08	0.055	90.91	38.70
	p_E		**0.039**	0.500	0.815			
	$p_{C/E}$							
aβ2GPI IgG or IgM or IgA	CLIA	#Y*	57.89	96.67	72.31	0.546	96.70	57.62
		W	51.32	97.62	67.80	0.489	97.50	52.56
	p_C		0.110	1.000	**0.028**			
	ELISA	W*	42.11	95.29	61.18	0.374	94.12	47.93
		A	31.79	97.78	56.43	0.296	96.00	46.07
	p_E		**0.003**	0.250	0.150			
	$p_{C/E}$		**<0.001**	1.000	**<0.001**			

(Continued)

TABLE 3 | Continued

			Sensitivity (%)	Specificity (%)	Accuracy (%)	Youden Index	PPV (%)	NPV (%)
aβ2GPI IgG or IgM	CLIA	#Y*	57.24	97.75	72.31	0.55	97.75	57.52
		W	51.32	97.65	67.93	0.489	97.50	52.87
	p_C		0.163	1.000	**0.038**			
	ELISA	W*	39.47	96.55	60.25	0.364	95.24	47.73
		A	29.14	98.89	55.19	0.28	97.78	45.41
	p_E		**0.003**	0.250	0.061			
	$p_{C/E}$		**<0.001**	1.000	**<0.001**			

P-values of sensitivity and specificity are calculated with McNemar test; significant results are marked bold.
*P_C, Comparison of CLIA results from different manufacturers. *Better results.*
*P_E, Comparison of ELISA results from different manufacturers. *Better results.*
$P_{C/E}$, Comparison of better CLIA and ELISA results. #Best results.
Odds ratios (ORs) with 95% confidence intervals (CIs) are shown.

numbers in **Figure 1**. Patients positive for antibodies fell above the dotted line, and the range of distribution varied due to use of both test methods and limitation of test range for different antibodies. In general, W-CLIA had the widest range of test distribution, while W-ELISA had the narrowest. For Y-CLIA, test range limitation influenced distribution for three autoantibodies. The results of primary or secondary APS patients were compared to other groups and illustrated. Overall, most test systems could distinguish between APS patients and HC, while little significant difference was observed between PAPS and SAPS groups. For different antibodies, four test systems showed different strengths of differential diagnosis. For instance, W-CLIA was best at discrimination for aCL IgG, while A-ELISA was best at aCL IgM. Additionally, distribution of aPLs among clinical groups with the largest number of patients (*i.e.*, thrombosis, pregnancy morbidity, and thrombocytopenia) was also illustrated in **Figure 2**.

Cross-Positivity Analysis for Four aPL in APS Patients

Among 152 patients, cross positivity for IgG or IgM of aCL and aβ2GpI for each of the four test systems were demonstrated with Venn diagrams (**Figure 3**). For aCL, 50 (32.9%) patients were tested positive for IgG or IgM by all systems. There were 12 (7.9%) patients who were tested positive only by Y-CLIA, and 13 (8.6%) were tested positive only by W-ELISA. Similarly, for aβ2GpI, 19 (12.5%) patients were test positive only by Y-CLIA, and seven (4.6%) were tested positive only by W-CLIA. When combining the positivity of aCL and aβ2GpI, Y-CLIA identified the most amount of positive patients (totally 102, 67.8%), with the highest level of patients distinguished only by the system (16, 10.5%).

Clinical Manifestations Prediction for the Test Systems

The correlation of different aPL levels by all four test systems with non-criteria clinical manifestations was further explored, with significant results presented in **Table 5**. Thrombocytopenia was associated with the greatest number of antibody positivity (aCL IgG by Y-CLIA, aCL IgM/aβ2GpI IgG/aβ2GpI IgM by W-CLIA, and aβ2GpI IgM by W-ELISA). Significant association was also observed for APSN, PVT, PE, DVT, and positivity of some autoantibodies by certain test systems. Little association was observed between IgA with any clinical features.

DISCUSSION

APS is an autoimmune disease featuring thrombosis and/or pregnancy morbidity which may lead to severe consequences. In order to accurately identify APS patients and provide timely clinical intervention, a detection system with high sensitivity and specificity is required. In this study, the diagnostic and analytic performances of four commercial assays were compared in detecting IgG/IgM/IgA for aCL and aβ2GPI antibodies. In brief, CLIA by YHLO Biotech Co. was considered as the system with the best predictive power, where 58.55 and 57.89% of APS patients were positive for aCL or aβ2GPI for at least one antibodies (IgG or IgM or IgA). Y-CLIA also identified the greatest number of patients (67.8%) positive for aCL or aβ2GpI IgG or IgM, with the highest level of patients distinguished only by the system (16, 10.5%). Nevertheless, for Y-CLIA, little correlation of antibodies' positivity result with thrombosis or pregnancy complication was observed. In addition, the greatest number of double/triple patients was detected by Y-CLIA. Concerning clinical manifestations, a significant association was observed between W-CLIA and TP/PE, Y-CLIA and TP, as well as combined results with TP/PE/thrombosis. Overall, CLIA showed better performance characteristics than traditional ELISA test systems.

Many previous studies have found poor agreement among different aPL assay platforms (5, 18), which may result from various factors. As shown in **Table 2**, depending on the coating method for solid phase, antibodies detected would either bind to cardiolipin or bind directly to β2GPI. In addition, different conjugates were applied for signal detection. A lack of universal internal standards for calibration further increased the chance of discrepancy. In addition, different cut-off values were chosen, as they stem from heterogenous reference sample

TABLE 4 | Comparison of the predictive power of aPL tests from different test systems for criterial manifestations.

			Thrombosis				Pregnancy morbidity		
			Sensitivity (%)	**Specificity (%)**	**Accuracy (%)**		**Sensitivity (%)**	**Specificity (%)**	**Accuracy (%)**
aCL IgG	CLIA	Y*	52.94	58.06	54	Y	48	44.07	45.87
		W	43.67	70.97	49.34	W*	38	61.02	50.46
	p_C		**0.007**	0.125	**0.001**		0.125	**0.006**	**0.001**
	ELISA	W	40.34	71	46.67	W	32	62.71	48.62
		A	38.98	67.74	44.97	A	38.78	66.1	53.7
	p_E		0.754	1	1		0.25	0.625	1
	$p_{C/E}$								
aCL IgM	CLIA	Y	16.81	83.87	30.66	Y	16	84.75	53.21
		W	14.29	87.1	29.33	W	12	84.75	51.37
	p_C		1	0.453	0.344		0.687	1	0.727
	ELISA	W	31.09	54.84	36	W	40	67.8	48.62
		A	7.63	87.1	24.16	A	10.2	89.83	53.7
	p_E		**<0.001**	**0.006**	**<0.001**		**<0.001**	**<0.001**	**<0.001**
	$p_{C/E}$								
aCL IgA	CLIA	Y*	22.69	74.19	33.33	Y	26	76.27	53.21
		W	12.61	80.65	26.67	W*	20	88.14	56.88
	p_C		**0.004**	0.5	**0.001**		0.375	**0.039**	**0.013**
	ELISA	W	2.52	87.1	20	W	8	96.61	55.96
		A	29.66	64.52	36.91	A	34.69	64.41	50.93
	p_E		**<0.001**	**0.016**	**<0.001**		**<0.001**	**<0.001**	**<0.001**
	$p_{C/E}$								
aCL IgG or IgM or IgA	CLIA	Y*	62.18	54.84	60.66	Y	52	35.59	43.12
		W	50.42	64.52	53.33	W*	42	52.54	47.71
	p_C		**0.001**	0.25	**<0.001**		0.125	**0.002**	**<0.001**
	ELISA	W	55.46	48.39	54	W	52	45.76	48.62
		A	53.39	51.61	53.02	A	51.02	49.15	50
	p_E		0.69	1	0.608		1	0.791	0.701
	$p_{C/E}$								
aCL IgG or IgM	CLIA	#Y*	62.18	54.84	60.66	Y	52	35.59	43.12
		W	50.42	67.74	54	W*	40	52.54	46.79
	p_C		**0.001**	0.125	**<0.001**		0.07	**0.002**	**<0.001**
	ELISA	W*	55.46	48.39	54	W	52	45.76	48.62
		A	43.22	64.52	47.65	A*	40.82	59.32	50.93
	p_E		**0.003**	0.18	**0.001**		0.18	**0.021**	**0.007**
	$p_{C/E}$		0.215	0.727	**0.022**		1	0.344	0.424
aβ2GPI IgG	CLIA	Y	47.9	54.84	49.33	Y	48	54.24	51.38
		W	53.78	61.29	55.34	W	46	42.37	44.04
	p_C		0.21	0.625	0.442		1	0.092	0.263
	ELISA	W*	33.61	74.19	42	W	26	67.8	48.63
		A	25.42	83.87	40.94	A*	22.45	79.66	53.71
	p_E		**0.021**	0.25	**0.004**		0.687	**0.016**	**0.022**
	$p_{C/E}$								
aβ2GPI IgM	CLIA	#Y*	22.69	83.87	35.33	Y	20	77.97	51.37
		W	8.4	87.1	24.67	W*	10	91.53	54.13
	p_C		**<0.001**	1	**<0.001**		0.125	**0.008**	**0.001**
	ELISA	W*	15.97	87.1	30.67	W	16	83.05	52.29
		A	8.47	93.55	26.17	A	6.12	93.22	53.71
	p_E		**0.039**	0.625	**0.004**		**0.031**	0.219	**0.006**
	$p_{C/E}$		0.077	1	**0.001**				
aβ2GPI IgA	CLIA	Y	14.29	74.19	26.66	Y*	26	88.14	59.64
		W	13.45	83.87	28	W	20	88.14	56.88
	p_C		1	0.375	0.344		0.453	1	**0.013**
	ELISA	W*	10.92	83.87	28.25	W	14	84.75	52.29
		A	6.78	93.55	24.83	A	6.12	89.83	51.85
	p_E		0.125	0.375	**0.039**		0.289	0.25	0.065
	$p_{C/E}$								
aβ2GPI IgG or IgM or IgA	CLIA	Y	60.5	48.29	58	Y	58	44.01	50.46
		W	55.46	61.29	56.67	W	46	42.37	47.71
	p_C		0.345	0.125	0.11		0.07	1	0.383
	ELISA	W	45.38	67.74	50	W	38	57.63	48.62
		A	34.75	77.42	57.63	A*	28.57	71.19	51.85

(Continued)

TABLE 4 | Continued

			Thrombosis				Pregnancy morbidity		
			Sensitivity (%)	Specificity (%)	Accuracy (%)		Sensitivity (%)	Specificity (%)	Accuracy (%)
	p_E		**0.008**	0.375	**0.003**		0.219	**0.002**	**0.002**
	$p_{C/E}$								
aβ2GPI IgG or IgM	CLIA	Y	60.5	51.61	58.67	Y	56	44.07	49.54
		W	55.46	61.29	56.67	W	46	42.37	44.04
	p_C		0.345	0.375	0.163		0.18	1	0.523
	ELISA	W*	49.58	64.52	52.66	W	40	50.85	45.87
		A	40.68	74.19	47.65	A*	28.57	61.02	46.29
	p_E		**0.021**	0.375	**0.007**		0.125	**0.031**	**0.003**
	$p_{C/E}$								

P-values of sensitivity and specificity are calculated with McNemar test; significant results are marked bold.
*P_C, Comparison of CLIA results from different manufacturers. *Better results.*
*P_E, Comparison of ELISA results from different manufacturers. *Better results.*
$P_{C/E}$, Comparison of better CLIA and ELISA results. #Best results.
Odds ratios (ORs) with 95% confidence intervals (CIs) are shown.

groups in the original calculation. Thus, it might be better to choose the same appropriate reference population among all platforms and utilize an in-house 99th percentile cut-off value, which had been recommended by all manufacturers and confirmed by previous studies (19, 20). Nevertheless, due to the restriction of subjects, this study still chose the cut-off values provided by platform instructions respectively, which might not reflect the distribution characteristics of the disease population. Compared to ELISA, automated CLIA has the advantage of increasing reproducibility, reducing hands-on time as well as avoiding manual error, which had been proved by some previous studies.

With regard to the predictive value of aPLs detected by the four systems, Y-CLIA stood out as the best. **Table 3** indicated that the sensitivity, specificity, accuracy, and Youden index were higher for Y-CLIA among each comparison whenever a significant difference was found. As for ELISAs, W-ELISA had higher predictive power for most aPLs compared to A-ELISA. However, no single detection system had stably shown better performance for all aPLs. Distribution of aPL test results in **Figure 1** further reflected this inconsistency. Y-CLIA did not show better ability at distinguishing PAPS or SPAS from SLE or HC groups compared to other systems. Indeed, it had been estimated in previous studies that around 40% of patients with SLE have aPL, and APS may develop in up to 50–70% of patients with both SLE and aPLs (21). Thus, although Y-CLIA could be recommended for APS diagnosis, other systems may provide additive value for each individual aPL in differentiation, especially when SLE was involved. The predictive power of criterial manifestations indicated that besides serology diagnosis, different systems had respective strengths in predicting associate events. W-CLIA was more sensitive and accurate for thrombosis, while results from A-ELISA were more specific and accurate for pregnancy-related outcomes. Since APS diagnosis relied both on clinical and experimental criteria, inclusion of more test systems was still of great importance.

As IgG or IgM of aCL and aβ2GpI was part of the standard diagnostic criteria, cross-positivity analysis was conducted, which revealed that Y-CLIA identified the most number of patients test positive overall. However, other systems were still of great value for different aPLs, as 8.6% of aCL and 4.6% of aβ2GpI were tested positive only by W-ELISA or W-CLIA. which suggested that a combination of more test systems could increase the sensitivity of APS diagnosis. In the clinic, patients may remain persistently negative for criteria aPLs yet show typical APS clinical manifestations (defined as seronegative APS, SNAPS) (22). Alternate testing platforms could assist in final diagnosis for SNAPS patients.

According to the European League Against Rheumatism (EULAR) guidelines for APS, high-risk profiles for APS is defined as a positive LA test, the presence of double (any combination of LA, aCL or aβ2GPI antibodies) or triple (all three subtypes) aPL positivity, or the presence of persistently high aPL titers (23). It is crucial to recognize these high-risk patients in order for the early prevention of thrombotic and obstetric events (24). Thus, a cross-positivity analysis was conducted to evaluate the ability of four test systems in identifying high-risk patients concerning aCL/aβ2GPI detection (result not shown). For double-positive patients, among 94 patients (61.84%) positive for LA and aCL, eleven and nine were detected positive only by Y-CLIA and W-ELISA respectively. Among 92 patients (60.53%) positive for LA and aβ2GPI, seven and six were detected positive only by Y-CLIA and W-CLIA respectively. For 77 triple-positive patients (50.66%), nine were detected positive only by Y-CLIA and two by W-CLIA. The result suggested that a combination of more test systems could increase the sensitivity of high-risk identification for APS.

Finally, the results of different aPL isotypes tested by four systems were explored of their association with non-criteria clinical manifestations. Thrombocytopenia was associated with the greatest number of antibody positivity, and significant results were also observed for APSN, PVT, PE, and DVT. However, no other significant association was observed for other clinical features or IgA isotype. Similar results could be observed in a study conducted by us recently in a large cohort with more than

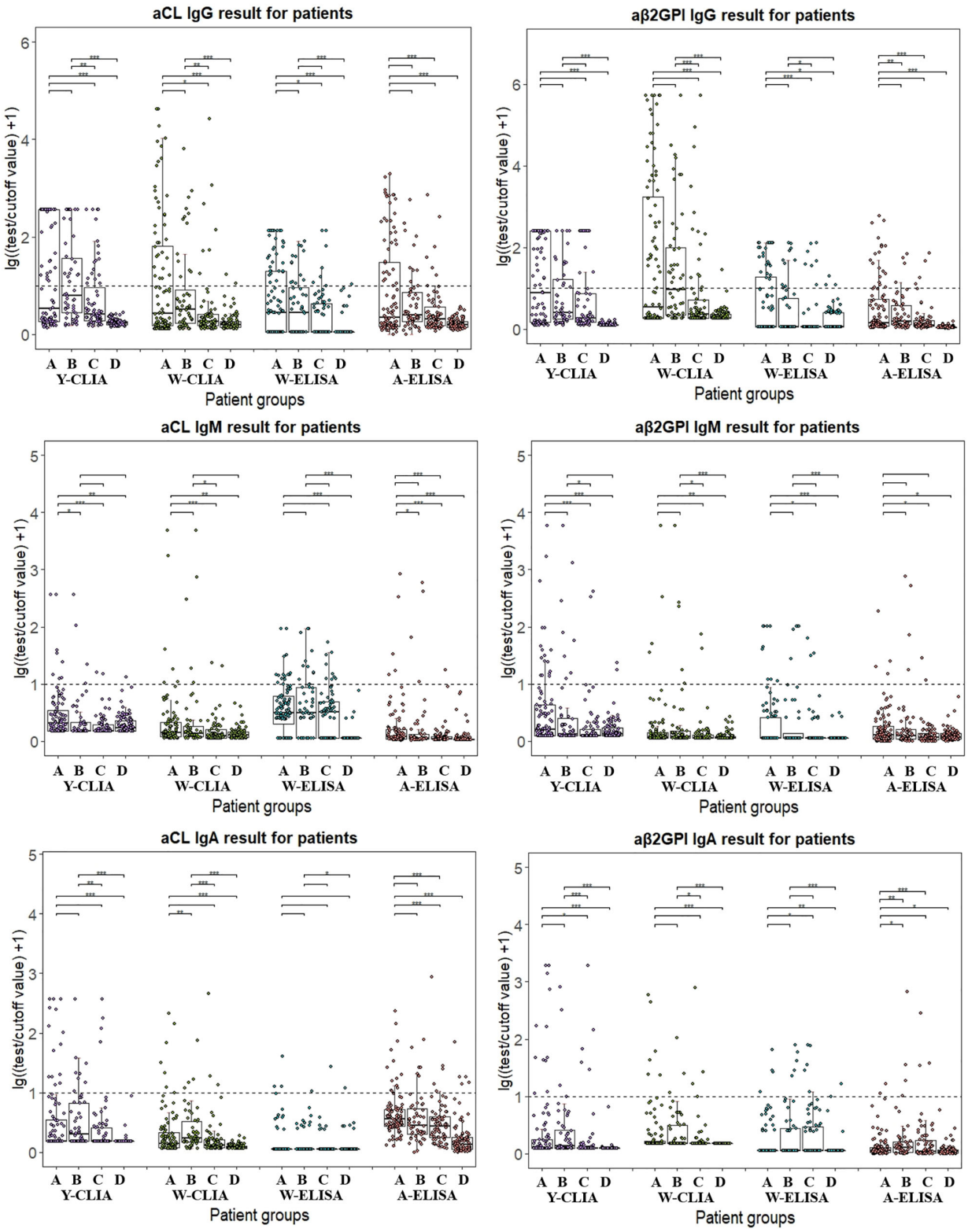

FIGURE 1 | Distribution of aPL test results from different manufacturers among different patient groups. Test results are calculated in lg[(test result/cutoff value) +1]. A: PAPS, B: SAPS, C: SLE, D: Health control. Wilcox's test is conducted comparing primary or secondary APS result to other patient groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

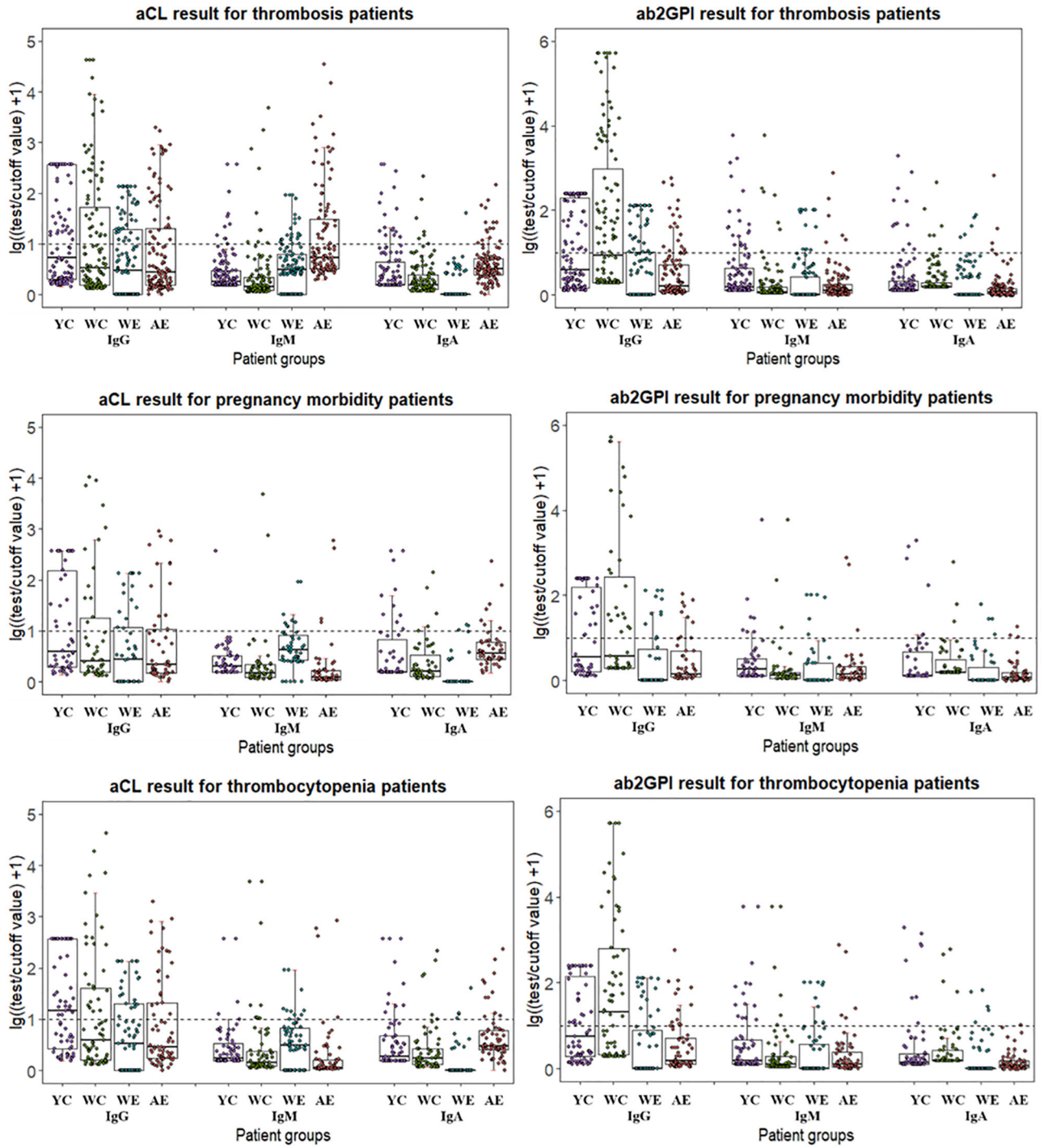

FIGURE 2 | Distribution of aPL test results from different manufacturers for patients with different manifestations. Test results are calculated in lg[(test result/cutoff value) +1]. YC, Y-CLIA; WC, W-CLIA; WE, W-ELISA; AE, A-ELISA.

7,000 patients (25). It had been reported that the prevalence of thrombocytopenia was 20 to 46% as a manifestation of primary APS, probably because aCL may bind activated platelet membranes and cause platelet destruction (26). Although the correlation between aPLs and thrombosis or pregnancy events has been confirmed by a number of studies (27–29), conflicting results have also been observed in other reports (9, 30). In our study, venous thrombosis events (PVT, PE, and DVT) showed

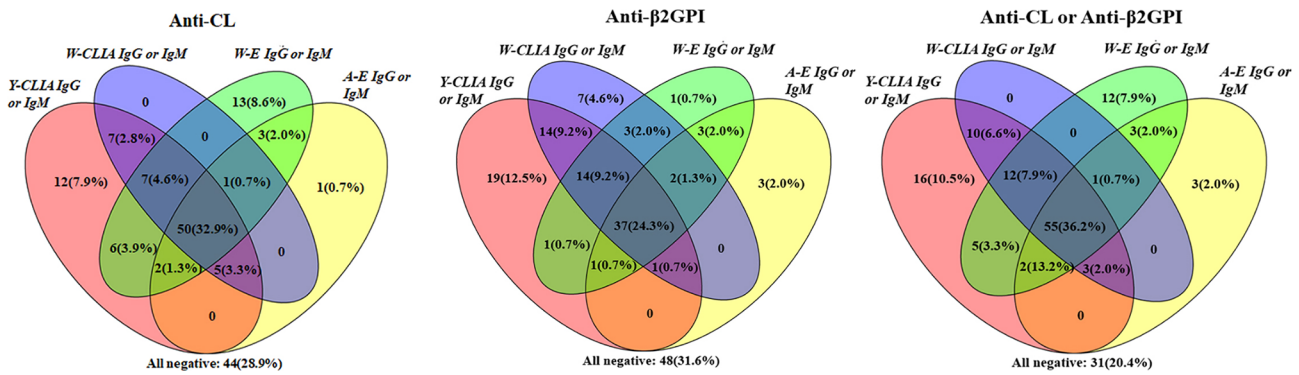

FIGURE 3 | Cross-positivity among different tests for anti-CL, anti-β2GPI, and anti-CL or anti-β2GPI antibodies in APS patients (n = 152).

TABLE 5 | Significant association between non-criteria clinical manifestations and aPL levels by different test systems.

	Significant association	χ^2	*p
YCLIA	TP & aCL IgG	6.935	0.008
	APSN & aβ2GPI IgG	5.644	0.026
	PVT & aCL IgG	5.308	0.027
WCLIA	PE & aCL IgG	7.636	0.006
	TP & aCL IgM	5.043	0.025
	TP & aβ2GPI IgG	4.511	0.034
	TP & aβ2GPI IgM	4.679	0.031
WELISA	TP & aβ2GPI IgM	4.913	0.027
	APSN & aβ2GPI IgG	7.369	0.013
	APSN & aCL IgG	9.007	0.005
AELISA	DVT & aβ2GPI IgM	5.466	0.035

**Fisher exact tests were conducted if needed. TP, thrombocytopenia; APSN, antiphospholipid syndrome nephropathy; PVT, portal vein thrombosis; PE, pulmonary emphysema; DVT, deep vein thrombosis.*

more correlation with aPL positivity, while little significant relationship was found with poor pregnancy outcomes. It should be noted that the number of patients with most of the recorded clinical manifestations was small (**Figure 1**). Consequently, the results might be strongly influenced by patient heterogeneity including age, gender, or other factors.

All in all, this study confirmed the advantage of using CLIA testing systems for aPL detection, with higher predictive power and better ability at identifying both low-titer suspected and multi-positive high-risk patients. In the future, with the reduction of test apparatus cost, fully automated CLIA could replace ELISA in the laboratory testing of aPLs for APS diagnosis and monitor. For the local population in China, Y-CLIA would be a more suitable choice concerning commercially available testing systems. Our study has some limitations. Recommended cut-off values were used and not calculated with the local population, which might decrease precision in sequential analysis. Correlation between autoantibodies and clinical manifestations, especially obstetrical related events, still needs examination. Larger sample size and inclusion of patients with a wider range of associated diseases or clinical features, as well as more high-risk patients (double/triple-positive), could further complement the study. The predictive performance of the selected test system (Y-CLIA) also needs further confirmation.

CONCLUSION

In conclusion, CLIA was considered a better platform for IgG/IgM/IgA aCL and aβ2GPI detection in APS diagnosis. Additionally, a combination of other detection platforms could assist in clinical diagnosis and differential diagnosis, increase the ability to exclude SNAPS, as well as identify high-risk patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Peking Union

Medical College Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors were involved in the design of this study. CH, SL, ZX, HY, HJ, and JZ contributed to the collection of blood samples and other experimental procedures. YS and WQ were involved in data collection and pre-processing. CH and SL analyzed the data and wrote the manuscript. JZ, QW, XT, ML, and YZ contributed to the recruitment of patients and evaluation of clinical data. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Urine Proteomics Differentiate Primary Thrombotic Antiphospholipid Syndrome From Obstetric Antiphospholipid Syndrome

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Antiphospholipid syndrome (APS) is a multisystem disorder characterized by thrombosis and/or recurrent fetal loss. This clinical phenotype heterogeneity may result in differences in response to treatment and prognosis. In this study, we aimed to identify primary thrombotic APS (TAPS) from primary obstetric APS (OAPS) using urine proteomics as a non-invasive method. Only patients with primary APS were enrolled in this study from 2016 to 2018 at a single clinical center in Shanghai. Urine samples from 15 patients with TAPS, 9 patients with OAPS, and 15 healthy controls (HCs) were collected and analyzed using isobaric tags for relative and absolute quantification (iTRAQ) labeling combined with liquid chromatography-tandem mass spectrometry analysis to identify differentially expressed proteins. Cluster analysis of urine proteomics identified differentiated proteins among the TAPS, OAPS, and HC groups. Urinary proteins were enriched in cytokine and cytokine receptor pathways. Representative secreted cytokines screened out (fold change >1.20, or <0.83, $p<0.05$) in these differentiated proteins were measured by enzyme-linked immunosorbent assay in a validation cohort. The results showed that the levels of C-X-C motif chemokine ligand 12 (CXCL12) were higher in the urine of patients with TAPS than in those with OAPS ($p=0.035$), while the levels of platelet-derived growth factor subunit B (PDGFB) were lower in patients with TAPS than in those with OAPS ($p=0.041$). In addition, correlation analysis showed that CXCL12 levels were positively correlated with immunoglobulin G anti-β2-glycoprotein I antibody ($r=0.617$, $p=0.016$). Our results demonstrated that urinary CXCL12 and PDGFB might serve as potential non-invasive markers to differentiate primary TAPS from primary OAPS.

Keywords: thrombotic antiphospholipid syndrome, obstetric antiphospholipid syndrome, CXCL12, PDGFB, urine proteomics

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INTRODUCTION

Antiphospholipid syndrome (APS) is a multisystem disorder characterized by a combination of arterial and/or venous thrombosis, recurrent fetal loss in women, and persistent presence of antiphospholipid antibodies (aPLs) including lupus anticoagulant (LAC), anti-β2-glycoprotein I (aβ2GPI) and anti-cardiolipin antibody (aCL) (1). APLs constitute a heterogeneous group of immunoglobulins directed against phospholipids or specific phospholipid-binding plasma proteins (2). The persistent presence of aPLs is recognized as an important laboratory diagnostic criterion for the definite classification of patients with APS according to the updated 2006 Sydney modification 'Sapporo criteria' (3). APS is a heterogeneous disease combined with thrombotic and obstetric complications (4). Genomic and proteomic studies have been carried out to explore the underlying mechanisms. For example, monocytes derived from patients with APS, patients with thrombosis without APS, and healthy controls (HCs) expressed different genes, such as annexin I and annexin II (5). Furthermore, IgG from patients with only thrombosis and only obstetric complications triggered different signaling pathways in monocytes (6). Another study also reported that being treated with thrombotic or obstetric APS IgG compared with HC IgG, four of the most significantly changed proteins in human monocytes were vimentin, myeloperoxidase, cytoskeleton-associated protein glycine-rich domain-containing linker protein 2, and zinc finger CCH domain-containing protein (7). According to these findings, thrombotic and obstetric APS may be two different subtypes. However, there are no biomarkers to differentiate TAPS from OAPS.

Urine proteomics has recently received increasing attention. Urine collection is an easy, non-invasive procedure. And it reflects the condition of the body in many aspects. It has been reported that urine proteins can serve as diagnostic biomarkers in rheumatic diseases. In systemic lupus erythematosus (SLE), urine proteins such as osteopontin N-half and urinary monocyte chemoattractant protein-1 could distinguish patients with lupus nephritis (LN) from those without LN (8, 9). Urinary cluster of differentiation was found to have a diagnostic value comparable to traditional serum biomarkers in rheumatoid arthritis (RA) (10). However, the role of urine proteomics in primary APS needs to be clarified. Therefore, we aimed to carry out urine proteomics in patients with primary APS to further identify TAPS from OAPS.

MATERIALS AND METHODS

Patients

Primary APS patients (PAPS) were enrolled consecutively from 2016 to 2018 in Department of Rheumatology and Immunology in Ruijin Hospital, Shanghai. Patients who met the criteria for the classification of PAPS using the Sydney criteria were included (3). The diagnosis of PAPS was confirmed by two senior rheumatologists: Jialin Teng and Chengde Yang. Any APS patients secondary to other diseases such as SLE were excluded from this study. Finally, 15 patients with TAPS, 9 patients with OAPS, and 15 HCs were enrolled in the test cohort. To exclude obstetric complications, female patients in TAPS group were all with successful obstetric outcome. OAPS patients without the history of thrombosis were included. All HCs were recruited from age- matched volunteers with no history of autoimmune, rheumatic, or other diseases. All the patients recruited in our study were treatment-naïve, without receiving any anticoagulant therapy. Proteomics analysis was performed by protocol as previously used by our group to identify differentially expressed proteins in the urine (11). In the validation cohort, urine samples of 19 patients with primary OAPS, 21 patients with primary TAPS, 13 HCs with positive aPL antibodies (APL carriers), 20 patients with miscarriages (non-autoimmune), 21 patients with thrombosis (non-autoimmune), 30 patients with SLE, 30 patients with RA and 30 HCs were tested by enzyme-linked immunosorbent assay (ELISA). RA patients who met 2010 Rheumatoid Arthritis Classification Criteria were included (12). SLE patients were diagnosed using 2012 Systemic Lupus International Collaborating Clinics (SLICC) classification criteria (13). Patients with 3 unexplained miscarriages of less than 10 weeks were regarded as miscarriage, while patients with at least one unexplained death of a morphologically normal fetus of over 10 weeks were regarded as intrauterine death, excluding other causes. Thrombosis was defined according to the established criteria, using laboratory, imaging or Doppler, or histopathologic data. APL carriers were defined as HCs with positive aPL antibodies (either aCL, aβ2GPI or LAC). According to the Sydney criteria, positive aCL and aβ2GPI antibodies were defined as IgG and IgM aCL in the serum or plasma presented at a medium or high titer (more than 40 GPL or MPL, or the 99th percentile), and IgG and IgM aβ2GPI at a titer over the 99th percentile. These antibodies were measured by ELISA (Euroimmun, Germany) at least twice and 12 weeks apart. LAC was measured by the Automated Coagulation Laboratory 300R (Milan, Italy) according to the criteria of the International Society on Thrombosis and Haemostasis (ISTH) committee (14). All patients were screened using the dilute Russellgically normal fetus (dRVVT) testing and the activated partial thromboplastin time. The ratio of the dRVVT screening time/dRVVT confirming time over 1.20 was considered as LAC positive. Hypocomplementemia was defined as a low serum level of complement 3 and/or complement 4. Thrombocytopenia was defined as numbers of platelets less than $100*10^9$/L. Proteinuria was defined as more than 500mg/24h urine protein. Medical documents and laboratory tests were collected through the electronic system. Urine samples were collected early morning from inpatients excluding urinary tract infections, and frozen at -80°C until testing. The detection and analysis of these specimens were performed at the same time in the same batch in the test study and validation study, respectively. The study was performed under the Declaration of Helsinki and the Principles of Good Clinical Practice and approved by the Institutional Review Broad of Ruijin Hospital (ID:2016-62), Shanghai Jiao Tong University School of

Medicine, Shanghai, China. Informed consent was obtained from the recruited subjects.

Urine Sample Processing

Taking the relatively low concentration of the protein levels into account, every 5 patients' urine samples were mixed into one, and analyzed SDT buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl, pH 8.0) was added to the sample and the lysate was boiled for 15 min. After centrifuged at 14000g for 40 min, the supernatant was quantified with the BCA Protein Assay Kit (Bio-Rad, USA). The proteins were separated on 12.5% SDS-PAGE gel. Protein bands were visualized by Coomassie Blue R-250 staining (Beyotime, Shanghai). 200 μg of proteins for each sample were incorporated into 30 μl SDT buffer. The detergent, DTT and other low-molecular-weight components were removed using UA buffer, which was 8 M Urea, 150 mM Tris-HCl pH 8.0, by repeated ultrafiltration (Microcon units, 10 kD, Germany). Then 100 μl iodoacetamide (100 mM IAA in UA buffer) was added to block reduced cysteine residues and the samples were incubated for 30 min in darkness. The filters were washed with 100 μl UA buffer three times and then 100 μl Dissolution buffer (DS buffer) twice. Finally, the protein suspensions were digested with 4 μg trypsin (Promega, USA) in 40 μl DS buffer overnight at 37°C, and the resulting peptides were collected as a filtrate. The peptides of each sample were desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 ml, Sigma, USA), concentrated by vacuum centrifugation and reconstituted in 40 μl of 0.1% (v/v) formic acid.

iTRAQ Labeling

100 μg peptide mixture of each sample was labeled using iTRAQ reagent according to the manufacturer's instructions (Applied Biosystems, USA). ITRAQ labeled peptides were fractionated by Strong Cation Exchange (SCX) chromatography using the AKTA Purifier system (GE Healthcare, USA). The dried peptide mixture was reconstituted and acidified with buffer A (10 mM KH_2PO4 in 25% of ACN, pH 3.0) and loaded onto a PolySULFOETHYL 4.6 x 100 mm column (5 μm, 200 Å, PolyLC Inc, Maryland, USA). The peptides were eluted at a flow rate of 1 ml/min with a gradient of 0%–8% buffer B (500 mM KCl, 10 mM KH_2PO4 in 25% of ACN, pH 3.0) for 22 min, 8–52% buffer B during 22-47 min, 52%-100% buffer B during 47-50 min, 100% buffer B during 50-58 min, and buffer B was reset to 0% after 58min. The elution was monitored by absorbance at 214 nm, and fractions were collected every 1 min. The collected fractions were desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 ml, Sigma), and concentrated by vacuum centrifugation.

LC-MS/MS Analysis

LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific, USA) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific, USA) for 60 min (determined by project proposal). The mass spectrometer was operated in positive ion mode. MS data were acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan (350-1800 m/z) for (high energy collision dissociation (HCD) fragmentation. The automatic gain control (AGC) target was set to 3e6, and maximum inject time to 50 ms. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 17,500 at m/z 200, and isolation width was 2 m/z. The normalized collision energy was 30 eV. All peptide ratios were normalized by the median protein ratio. The median protein ratio should be 1 after the normalization.

Enzyme-Linked Immunosorbent Assay

The urine concentration of C-X-C motif chemokine ligand 12 (CXCL12 or stromal cell-derived factor 1, SDF-1), platelet-derived growth factor subunit B (PDGFB), matrix metalloproteinase3 (MMP3), and platelet-derived growth factor receptor alpha (PDGFRA) were measured by human ELISA assay kit (R&D, USA). ELISA was performed according to the manufacture's instruction.

Statistical Analysis

Blast2GO was used in gene ontology (GO) enrichment analysis to annotate the target protein set, and the bar graph was drawn. Cluster 3.0 was used in cluster analysis. R language pack was used to make the Venn diagram and volcano plots. All data were statistically analyzed using the Statistical Package for the Social Sciences for Windows (V.23.0; SPSS, IBM). Graphs were drawn using GraphPad Prism software 8.0 (GraphPad Software, USA). Quantitative data between two groups with a Gaussian distribution were analyzed using an unpaired *t*-test. ANOVA was performed to compare the differences among multiple groups. *Chi*-square test and Kruskal-Wallis test were carried out for comparisons of two or more than two groups with categorical variable, respectively. Spearman rank-order correlation analysis was performed to calculate the correlation coefficient and *p* value between specific protein and aPLs. Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to access the sensitivity and specificity. In the test study, fold change of more than 1.2 or less than 0.83 was considered significant. $P<0.05$ was considered statistically significant.

RESULTS

Clinical Characteristics of Patients With APS

The clinical characteristics of patients with primary APS and HCs enrolled in the test cohort were presented in **Table 1**. Fifteen patients with TAPS, 9 patients with OAPS, and 15 HCs were included. Then, 19 patients with primary OAPS, 21 patients with primary TAPS, 13 APL carriers, 20 patients with miscarriages (non-autoimmune), 21 patients with thrombosis (non-autoimmune), 30 patients with SLE, 30 patients with RA,

TABLE 1 | Clinical and laboratory features of patients with TAPS, OAPS and HCs in the iTRAQ study.

	TAPS (n=15)	OAPS (n=9)	HCs (n=15)	*P* value
Age (mean ± SD)	41.1 ± 15.3	34.8 ± 5.0	42.1 ± 14.1	0.402
Sex (female/male)	10/5	9/0	13/2	0.054
Duration (months)	2.1 ± 2.1	4.3 ± 3.7	/	0.125
Venous thrombosis	15 (100.0%)	0 (0.0%)	/	<0.001
Arterial thrombosis	5 (33.3%)	0 (0.0%)	/	0.118
Miscarriage, <10 weeks	0 (0.0%)	6 (66.7%)	/	0.001
Intrauterine death, >10 weeks	0 (0.0%)	4 (44.4%)	/	0.012
LAC positive	10 (66.7%)	6 (66.7%)	/	1.000
IgG aCL positive	4 (26.7%)	3 (33.3%)	/	1.000
IgM aCL positive	2 (13.3%)	1 (11.1%)	/	1.000
IgG aβ2GPI positive	4 (26.7%)	3 (33.3%)	/	1.000
IgM aβ2GPI positive	1 (6.7%)	3 (33.3%)	/	0.130
ANA positive	0 (0.0%)	0 (0.0%)	/	/
Anti-dsDNA positive	1 (6.7%)	1 (11.1%)	/	1.000
Hypocomplementemia	3 (20.0%)	4 (44.4%)	/	0.356
Thrombocytopenia	7 (46.7%)	3 (33.3%)	/	0.678
ESR (mm/h)	38.2 ± 42.5	19.1 ± 12.5	/	0.233
aGAPSS	8.3 ± 4.2	9.7 ± 3.9	/	0.447
Proteinuria	1 (6.7%)	1 (11.1%)	/	1.000

iTRAQ, isobaric tags for relative and absolute quantification; TAPS, thrombotic antiphospholipid syndrome; OAPS, obstetric antiphospholipid syndrome; HCs, healthy controls; SD, standard deviation; LAC, lupus anticoagulant; aCL, antibodies to cardiolipin; aβ2GPI, antibodies to β2-glycoprotein I; ESR, erythrocyte sedimentation rate; aGAPSS, adjusted Global Antiphospholipid Syndrome Score.

and 30 HCs were included in the validation cohort, and the clinical data were shown in **Table 2**.

Comparison of Patients With TAPS, Patients With OAPS and HCs in the iTRAQ Study

To identify proteins that were differentially expressed in primary APS (TAPS and OAPS) and HCs, iTRAQ was performed on the urine in these samples. Data interpretation was divided into three groups: TAPS *vs.* HCs, OAPS *vs.* HCs, and OAPS *vs.* TAPS. Cluster analysis (**Figures 1**, **2** and **3A**) demonstrated that there were 36 upregulated proteins and 392 downregulated proteins, which added up to a total of 428 proteins in TAPS *vs.* HCs. In addition, there were 132 upregulated proteins and 337 downregulated proteins, which summed up to 469 proteins in OAPS *vs.* HCs. Furthermore, there were 349 upregulated proteins and 147 downregulated proteins, which added up to 496 proteins in OAPS *vs.* TAPS (**Supplementary Table 1**). Moreover, in the Venn diagram (**Supplementary Figure 1**), a total of 136 proteins changed in TAPS *vs.* HCs, 236 proteins in OAPS *vs.* HCs, and 211 proteins in OAPS *vs.* TAPS. Proteins with fold change values >1.2 or <0.83, and p values <0.05, were selected for further screening. The 10 most secreted urine proteins were identified as potential biomarkers among the TAPS, OAPS, and HC groups (**Supplementary Tables 2–4**). GO analysis comprised three parts: biological processes, molecular functions, and cellular components (**Figures 1–3C**). It indicated that cytokines and cytokine receptors were present, as reported in APS, which might play an important role (15). Then, CXCL12, PDGFB, MMP3 and PDGFRA screened out in the iTRAQ study were validated by ELISA in the validation cohort.

TABLE 2 | Clinical and laboratory features of patients with TAPS, patients with OAPS, APL carriers, patients with miscarriages, patients with thrombosis, SLE, RA and HCs in the validation cohort.

	TAPS (n=21)	OAPS (n=19)	APL carriers (n=13)	Miscarriages (n=20)	Thrombosis (n=21)	SLE (n=30)	RA (n=30)	HCs (n=30)	*P* value
Age (Mean ± SD)	40.9 ± 11.1	35.6 ± 8.6	38.4 ± 16.0	32.0 ± 4.7	40.2 ± 12.1	31.6 ± 5.1	36.4 ± 14.5	35.5 ± 8.2	0.063
Sex (female/male)	13/8	19/0	11/2	20/0	13/8	27/3	25/5	19/11	0.001
Duration(months)	3.8 ± 6.5	4.7 ± 6.8	/	/	/	2.8 ± 2.6	2.7 ± 2.2	/	0.654
Venous thrombosis	21 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	9 (42.9%)	/	/	/	<0.001
Arterial thrombosis	6 (28.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	12 (57.1%)	/	/	/	<0.001
Miscarriage, <10 weeks	0 (0.0%)	9 (47.4%)	0 (0.0%)	20 (100.0%)	/	/	/	/	<0.001
Intrauterine death, >10 weeks	0 (0.0%)	14 (73.7%)	0 (0.0%)	0 (0.0%)	/	/	/	/	<0.001
LAC positive	12 (57.1%)	15 (78.9%)	9 (69.2%)	/	/	/	/	/	0.341
IgG aCL positive	7 (33.3%)	8 (42.1%)	5 (38.5%)	/	/	/	/	/	0.850
IgM aCL positive	2 (9.5%)	1 (5.3%)	5 (38.5%)	/	/	/	/	/	0.025
IgG aβ2GPI positive	4 (19.0%)	6 (31.6%)	2 (15.4%)	/	/	/	/	/	0.500
IgM aβ2GPI positive	1 (4.7%)	4 (21.1%)	0 (0.0%)	/	/	/	/	/	0.091
ANA positive	0 (0.0%)	0 (0.0%)	/	/	/	30 (100.0%)	0 (0.0%)	/	<0.001
Anti-dsDNA positive	3 (14.3%)	3 (15.8%)	/	/	/	29 (96.7%)	0 (0.0%)	/	<0.001
Hypocomplementemia	6 (28.6%)	8 (42.1%)	/	/	/	25 (83.3%)	/	/	<0.001
Thrombocytopenia	10 (47.6%)	5 (26.3%)	/	/	/	8 (26.7%)	/	/	0.227
ESR (mm/h)	30.9 ± 37.9	24.0 ± 19.8	/	/	/	60.2 ± 37.9	20.6 ± 17.5	/	<0.001
aGAPSS	8.7 ± 3.9	9.8 ± 3.7	/	/	/	/	/	/	0.356
Proteinuria	2 (9.5%)	1 (5.3%)	/	/	/	14 (46.7%)	0 (0.0%)	/	<0.001

TAPS, thrombotic antiphospholipid syndrome; OAPS, obstetric antiphospholipid syndrome; APL, antiphospholipid antibody; SD, standard deviation; LAC, lupus anticoagulant; aCL, antibodies to cardiolipin; aβ2GP1, antibodies to β2-glycoprotein I; ESR, erythrocyte sedimentation rate; aGAPSS, adjusted Global Antiphospholipid Syndrome Score; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; HCs, healthy controls.

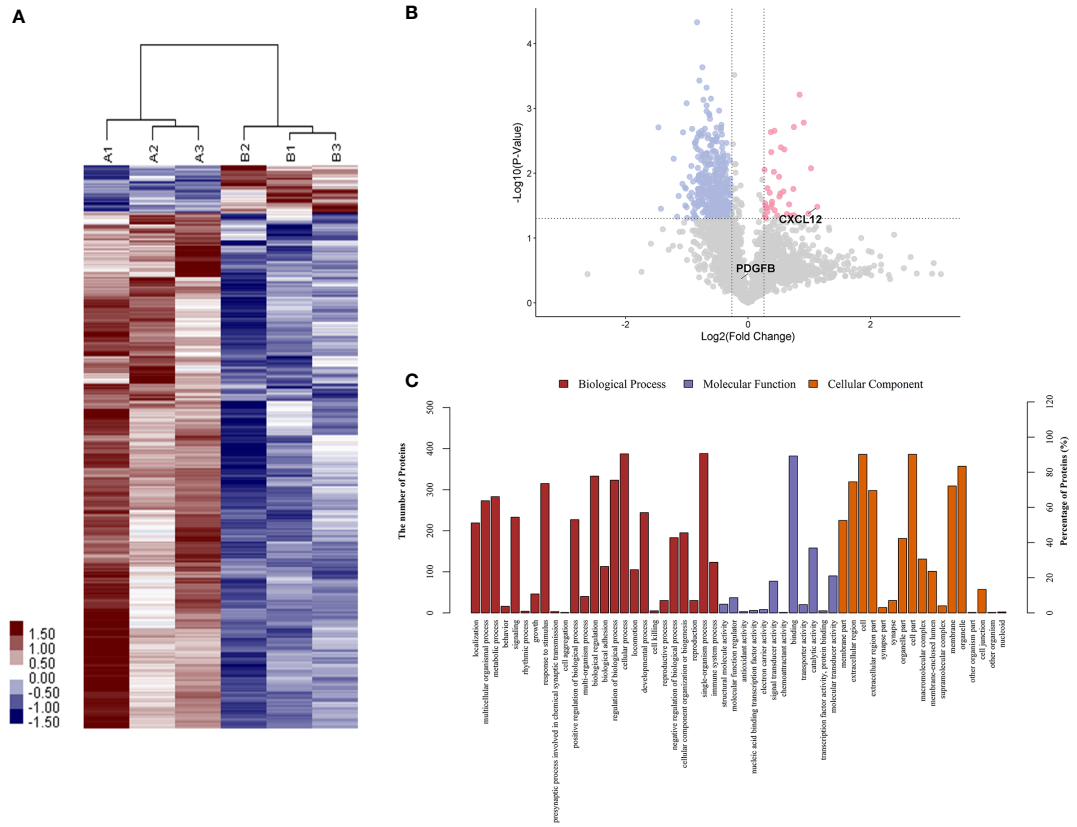

FIGURE 1 | The differentiated urine proteins between patients with TAPS and HCs analyzed by iTRAQ study. **(A)** Cluster analysis of patients with TAPS with HCs (A represented HCs; B represented TAPS). **(B)** Volcano plot showed that CXCL12 was in the upper right quadrant, which was in pink color area. **(C)** Gene ontology (GO) analysis of TAPS *vs* HCs. iTRAQ, isobaric tags for relative and absolute quantification; TAPS, thrombotic antiphospholipid syndrome; HCs, healthy controls.

Confirmation of CXCL12 and PDGFB in the Urine of Patients With TAPS and OAPS by ELISA in the Validation Cohort

In the validation study, urine samples from 19 patients with primary OAPS, 21 patients with primary TAPS, 13 APL carriers, 20 patients with miscarriages (non-autoimmune), 21 patients with thrombosis (non-autoimmune), 30 patients with RA, 30 patients with SLE, and 30 HCs were tested by ELISA. As shown in **Figure 4**, CXCL12 levels were higher in the urine of patients with TAPS than in those with OAPS (p=0.0350), APL carriers (p=0.0008), patients with miscarriages (p<0.0001), patients with thrombosis (p<0.0001), patients with RA (p=0.0012), patients with SLE (p<0.0001), and HCs (p<0.0001). CXCL12 levels were higher in patients with OAPS than in APL carriers (p=0.0123), patients with miscarriages (p<0.0001), patients with thrombosis (p=0.0024), and HCs (p=0.0007), but showed no differences compared to patients with RA (p=0.2510) or SLE (p=0.0791). On the other hand, PDGFB levels were higher in the urine of patients with OAPS than in those with TAPS (p=0.0406), APL carriers (p=0.0207), patients with miscarriages (p<0.0001), patients with thrombosis (p<0.0001), patients with RA (p=0.0002), patients with SLE (p=0.0052), and HCs (p=0.0005). Urinary PDGFB levels were higher in patients with TAPS than in those with RA (p=0.0425), with miscarriages (p=0.0144) and with thrombosis (p=0.0007). In addition, PDGFB levels in patients with TAPS was not significantly different from SLE (p=0.5294) or HCs (p=0.2067). In ROC curves, TAPS *vs*. HCs showed a higher AUC of 0.9328 (95% confidence interval [CI]: 0.8670-0.9987) (p<0.0001) than that of OAPS *vs*. HCs, which was 0.7867 (95% CI: 0.6375-0.9358) (p=0.0009) in urinary CXCL12 levels. In terms of urinary PDGFB levels, the AUC of OAPS *vs*. HCs was higher (AUC: 0.7886, 95% CI: 0.6594-0.9178) (p=0.0007) than that of TAPS *vs*. HCs (AUC: 0.6056, 95% CI: 0.4434-0.7678) (p=0.2031). In addition, there were no differences in the MMP3 (p=0.449) and PDGFRA (p=0.217) levels between OAPS and TAPS (**Supplementary Figure 2**).

Comparison of CXCL12, PDGFB and Clinical Features in the Validation Cohort

Both the iTRAQ study and validation test indicated the specificity of CXCL12 and PDGFB in differentiating OAPS from TAPS. To better understand the relationship between CXCL12, PDGFB, and the two APS subgroups, we further analyzed the correlation between the CXCL12, PDGFB, and aPL levels with different clinical characteristics. First, Spearman rank-order correlation

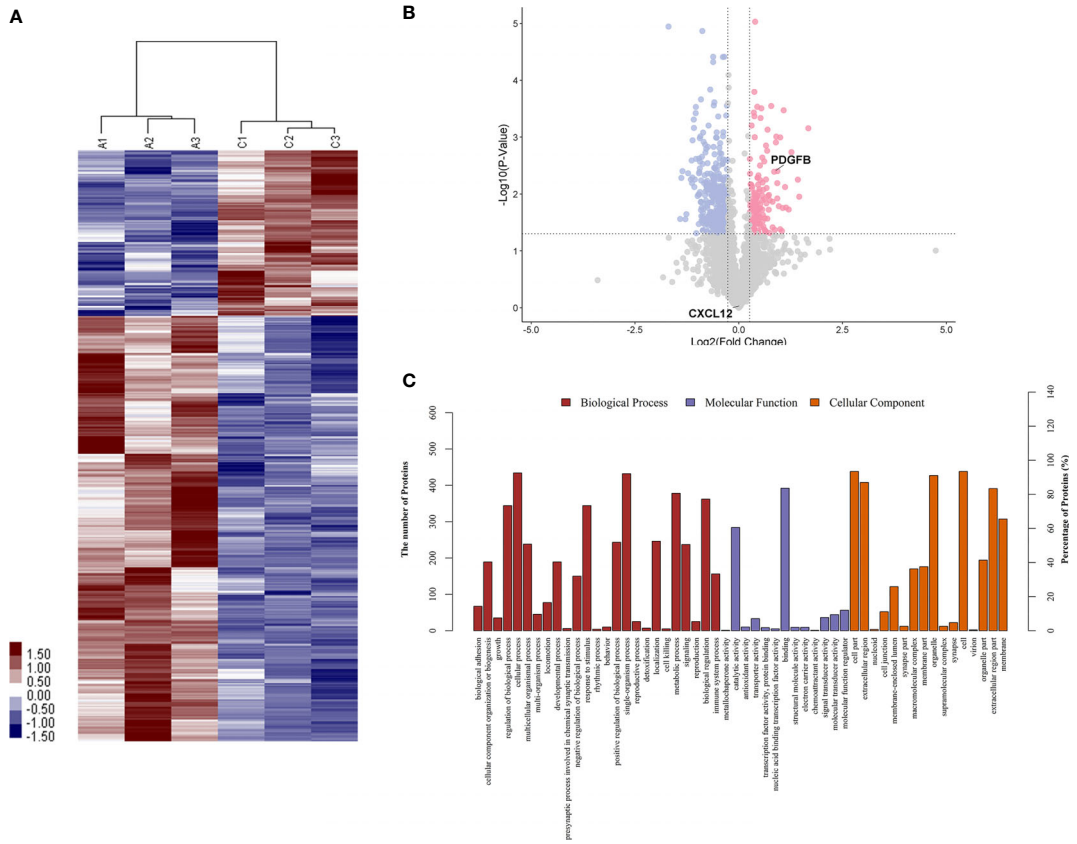

FIGURE 2 | The differentiated urine proteins between patients with OAPS and HCs analyzed by iTRAQ study. **(A)** Cluster analysis of patients with OAPS with HCs (A represented HCs; C represented OAPS). **(B)** Volcano plot showed that PDGFB was in the upper right quadrant, which was in pink color area. **(C)** Gene ontology (GO) analysis of OAPS *vs* HCs. iTRAQ, isobaric tags for relative and absolute quantification; OAPS, obstetric antiphospholipid syndrome; HCs, healthy controls.

analysis was used to evaluate the correlation between the levels of CXCL12, PDGFB, and aPLs (aCL, aβ2GPI, and LAC), respectively. It showed that CXCL12 levels were positively correlated with IgG aβ2GPI antibody (r=0.617, p=0.016) (**Figure 5**). In addition, there were no differences between the CXCL12 and PDGFB levels in patients with OAPS and TAPS with single, double, triple positive aPLs (**Supplementary Figure 3**). In patients with OAPS, the CXCL12 and PDGFB levels were analyzed according to the number of adverse pregnancy outcomes, and no differences were observed (**Supplementary Figure 4**). Moreover, in TAPS, the CXCL12 and PDGFB levels were also analyzed according to the number of thromboses and the presence or absence of arterial thrombosis, which indicated that there were no differences (**Supplementary Figure 5**).

DISCUSSION

Urine biomarkers have been widely discussed in recent years. It has been reported that there were more than 2300 proteins in the urine (16). It has also been reported that neutrophil gelatinase-associated lipocalin (NGAL), high mobility group box-1 (HMGB-1), vascular cell adhesion molecule-1 (VCAM-1), vitamin D-binding protein (VDBP) levels showed diagnostic performance for discriminating patients with LN from those without LN (17–20). Besides, collecting urine samples is more convenient and causes less pain in patients than while collecting blood samples. We previously reported a study in adult-onset Still's disease (AOSD) and found that urine α-1-acid glycoprotein 1 (LRG1), orosomucoid 1 (ORM1), and ORM2 might be new biomarkers of AOSD (11). Similarly, in this study, we analyzed urine proteomics to identify new biomarkers that might help differentiate TAPS from OAPS.

We used iTRAQ to screen for different proteins in the urine of patients with TAPS, patients with OAPS, and HCs. The results demonstrated that TAPS *vs*. HCs, OAPS *vs*. HCs, and OAPS *vs*. TAPS exhibited varied panels of upregulated and downregulated proteins, in which the CXCL12 levels were higher in the urine of those with TAPS than OAPS, while the PDGFB levels were lower in the urine of those with TAPS than OAPS. Then, CXCL12 and PDGFB levels were validated by ELISA in the validation cohort. Besides, we analyzed the correlation between CXCL12, PDGFB, and aPLs. The CXCL12 levels were positively correlated with IgG

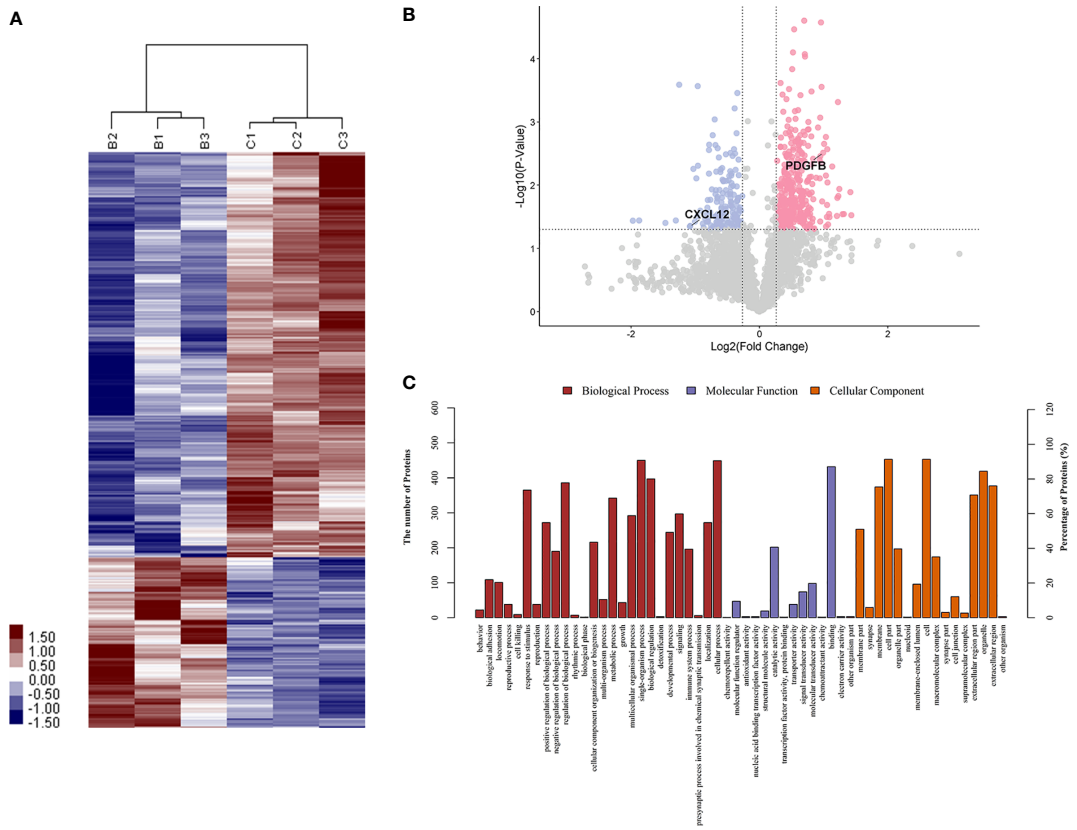

FIGURE 3 | The differentiated urine proteins between patients with OAPS and patients with TAPS analyzed by iTRAQ study. **(A)** Cluster analysis of OAPS with TAPS (B represented TAPS; C represented OAPS). **(B)** Volcano plot showed that PDGFB was in the upper right quadrant, which was in pink color area. CXCL12 was in the upper left quadrant, which was in blue color area. **(C)** Gene ontology (GO) analysis of OAPS *vs* TAPS. iTRAQ, isobaric tags for relative and absolute quantification; OAPS, obstetric antiphospholipid syndrome; HCs, healthy controls.

aβ2GPI antibody. Many previous studies manifested that cytokine played an important role in autoimmune diseases (21, 22). Thus, we investigated this aspect and assumed that CXCL12 and PDGFB might have the potential to differentiate TAPS from OAPS.

CXCL12 has been reported as stromal cell-derived factor-1 (SDF-1), a chemokine that plays an important role in the regulation of migration, proliferation, and differentiation of hematopoietic cells (23). CXCL12 fulfills its functions in homeostatic and pathological conditions by interacting with its receptors C-X-C chemokine receptor 4 (CXCR4) and atypical chemokine receptor 3 (ACKR3). Imbalances in the CXCL12/CXCR4/ACKR3 axis are associated with diseases, including cancer, multiple sclerosis, and RA (23). In addition, it has been reported that plasma CXCL12 levels were significantly elevated in the blood of patients with TAPS regardless of the arterial/venous nature of the thrombosis compared with HCs (24), which was similar to the results of our study. CXCL12 increases the chemotaxis of inflammatory cells and contributes to the activation of platelets in the damaged area, which could result in thrombosis or acceleration of the damage to the vascular integrity (25, 26). These studies demonstrated that CXCL12 might play an important role in the pathogenesis of TAPS. As reported previously, the functional role of the CXCL12 801 genotype involves the upregulation of CXCL12 protein. In a genomic study, patients with SLE with and without APS demonstrated different distributions of CXCL12 G801A genotype frequencies. SLE patients with APS demonstrated an increased frequency of the CXCL12 A allele and AA genotype compared with patients without APS, suggesting the clinical relevance of this polymorphism (27). It is promising that blockade of chemokine ligand 2 and CXCL12 could be as effective as cyclophosphamide in suppressing proliferative LN (28). It has also been indicated that the CXCR4-CXCL12 axis could be regarded as a potential therapeutic target because of its importance for antibody-secreting cells' homing and survival in lupus-prone mice (29).

PDGFB is mainly expressed in vascular endothelial cells, megakaryocytes, and neurons. PDGFB produced by endothelial cells drives the proliferation and spreading of vascular smooth muscle cells and pericytes in conjunction with angiogenesis (30). In addition, PDGFR-β and PDGFB appear to play a role in neuronal cardiac neural crest development, as both PDGFR-β and PDGFB knockout mice displayed abnormal cardiac innervation

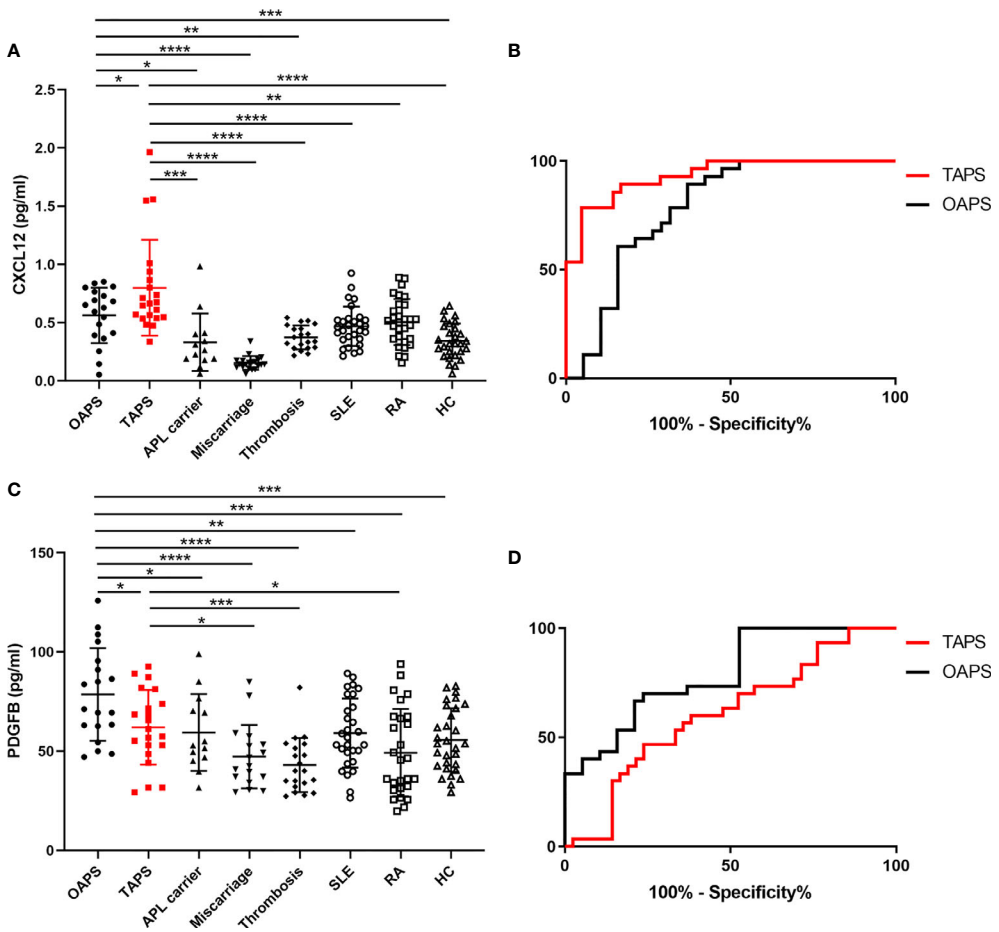

FIGURE 4 | Validation of CXCL12 and PDGFB as urinary biomarkers for patients with OAPS and patients with TAPS, respectively. **(A)** CXCL12 levels in the urine of patients with OAPS, patients with TAPS, APL carriers, patients with miscarriages, patients with thrombosis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and healthy controls (HCs). **(B)** The ROC curves of CXCL12 levels in the urine of TAPS *vs* HCs and that of OAPS *vs* HCs. **(C)** PDGFB levels in the urine of OAPS, TAPS, APL carriers, patients with miscarriages, patients with thrombosis, SLE, RA and HCs. **(D)** The ROC curves of PDGFB levels in the urine of TAPS *vs* HCs and that of OAPS *vs* HCs. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(30). Moreover, it has been reported that the toll-like receptor 9 (TLR9)/transforming growth factor-β1 (TGF-β1)/PDGFB pathway can be activated in SLE. Elevated PDGFB might contribute to the proliferation of renal mesangial cells and may be involved in the development of LN (31). However, more studies are needed to clarify the role of PDGFB in patients with APS.

Apart from these two cytokines, other biomarkers of APS have also been reported. Surface-enhanced laser desorption/ionization-time of flight analysis made it possible to discriminate between several proteins in women with pregnancy morbidity with and without aPLs, in which nine proteins were found in significantly higher levels in aPL-positive women (32). Apolipoprotein H and mitogen-activated protein kinase, previously described in the pathogenesis of APS, were found to differ in APS from non-APS patients with thrombosis (33). Patients with OAPS may also develop thrombosis. A total of 63% of women with OAPS developed thrombosis after initial obstetric morbidity. Women with subsequent thrombosis after OAPS had a higher adjusted global APS score (aGAPSS) (34).

There are several limitations to this study. First, the number of patients with primary APS was small. We included treatment-naïve patients with primary APS, which added the difficulty of recruiting patients. A larger group of patients are needed to verify the results. Second, not all patients in the TAPS group were females. There were no differences in the urinary CXCL12 and PDGFB levels between male and female in patients with TAPS (p>0.05) (**Supplementary Figure 6**). Selecting only female patients with TAPS for this study might cause selection bias. Therefore, we did not exclude male patients with TAPS.

In conclusion, the iTRAQ analysis showed that TAPS and OAPS distributed different urine protein patterns. Results of the validation study indicated that urinary CXCL12 and PDGFB might serve as potential biomarkers to differentiate primary TAPS from primary OAPS.

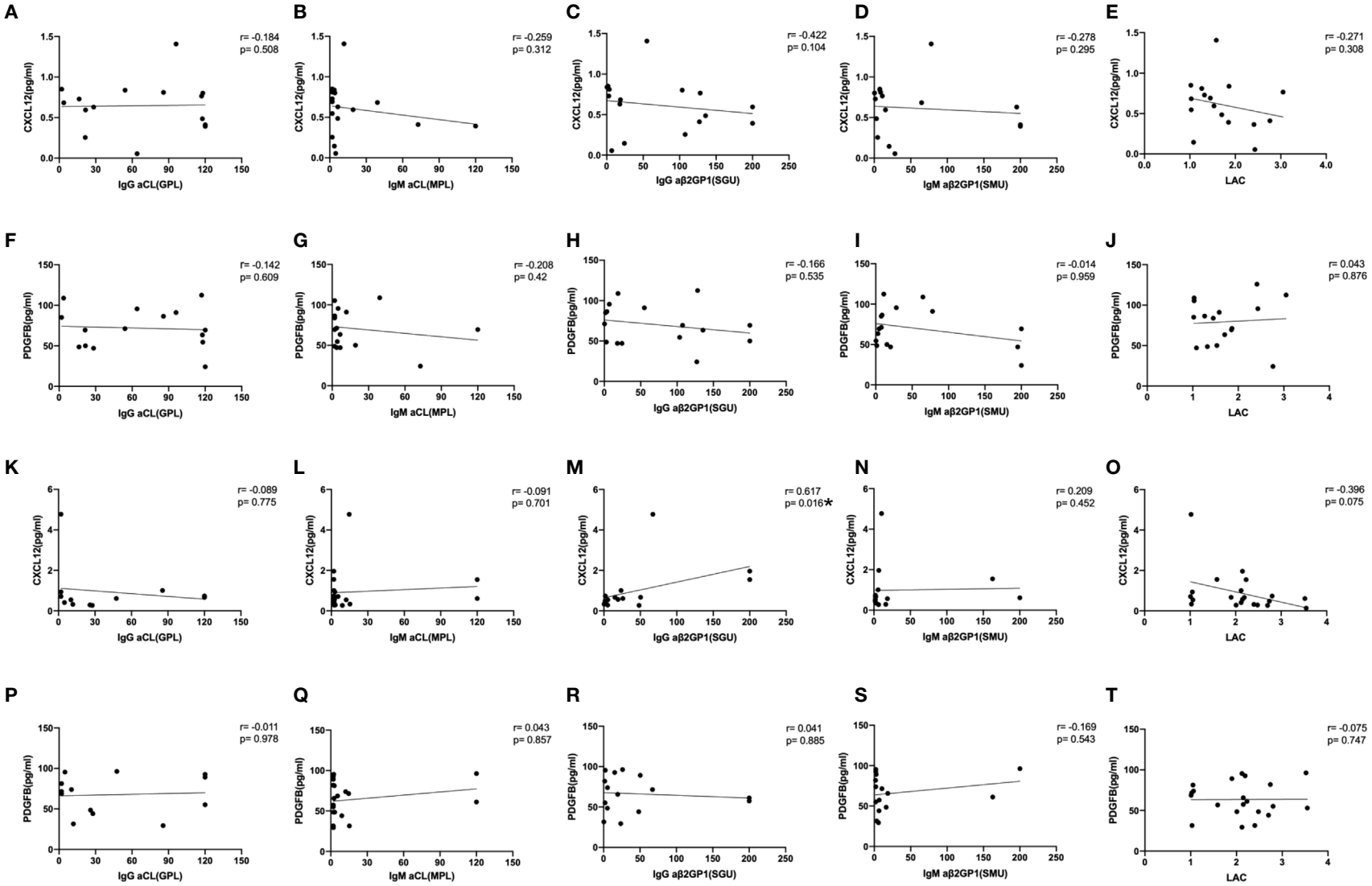

FIGURE 5 | The correlation between the levels of CXCL12, PDGFB with aPLs. The spearman rank-order correlation between the levels of CXCL12, PDGFB and anti-cardiolipin antibody (aCL), anti-β2-glycoprotein I antibody (aβ2GPI) and lupus anticoagulant (LAC) in OAPS patients **(A–J)** and TAPS patients **(K–T)**. *$p < 0.05$.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the iProX repository, accession number: IPX0003233000.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Broad of Ruijin Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CS, CY and JY conceived of the study and participated in its design and coordination. ZZ, YY and FW carried out the ELISA. YS, JT, HL, XC, YTS, HS, LW, TL, MW collected samples and contributed to data acquisition, analysis, and critical review for intellectual content. QH, HC and JJ performed the statistical analyses for all the data. ZZ, JY, YY, and FW drafted the manuscript and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.702425/full#supplementary-material

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METHODS
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A Novel ELISA Assay for the Detection of Anti-Prothrombin Antibodies in Antiphospholipid Syndrome Patients at High Risk of Thrombosis

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Autoantibodies targeting prothrombin (aPT) can be found in antiphospholipid syndrome (APS) patients. However, their detection has proven difficult to standardize. Here, we developed a new ELISA assay to improve the identification of aPT and compared its performance with currently available anti-phosphatidylserine/prothrombin antibodies (aPS/PT) and autoantibodies targeting prothrombin bound to the plastic plate (aPT-A) assays using a cohort of 27 APS patients at high risk of thrombosis. We generated a novel prothrombin variant, ProTS525A-Biot, carrying an artificial tag at the C-terminus suitable for site-specific biotinylation and added the mutation S525A to improve stability. ProTS525A-Biot was immobilized to neutravidin-coated plates at the desired density and with a defined orientation, i.e., pointing the N-terminal fragment-1 toward the solvent. Antibodies against ProTS525A-Biot (aPT-Bio) were found in 24 out of 27 triple-positive APS patients (88%). When compared to aPS/PT and aPT-A, aPT-Bio showed an excellent linear correlation with aPS/PT (R^2 = 0.85) but not with aPT-A (R^2 = 0.40). Since aPS/PT but not aPT-A are an emerging biomarker of thrombosis in APS, this method may find utility for detecting pathogenic aPT in APS but also other prothrombotic conditions such as COVID-19.

Keywords: antiphospholipid antibody syndrome, autoimmunity, acquired coagulation disorders, lipid-protein interaction, single-molecule biophysics, COVID-19

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INTRODUCTION

Antiphospholipid syndrome is a debilitating condition characterized by vascular thrombosis in the presence of antiphospholipid antibodies (aPL), which persist >12 weeks in patients' plasma (1). Among aPL, anti-prothrombin antibodies (aPT) are believed to be clinically relevant since they are linked to thrombosis (2). Currently, aPT are detected using two methods (3). First, prothrombin is directly immobilized onto a hydrophilic plastic plate, in which case aPT are named aPT-A. Alternatively, prothrombin is bound, in the presence of calcium ions, to plastic wells pre-coated with phosphatidylserine (PS), in which case aPT are referred as to anti-phosphatidylserine/prothrombin antibodies (aPS/PT). Beyond the technical aspects, this distinction has strong

clinical value since aPS/PT but not aPT-A are the ones often found in APS patients at high risk of thrombosis (3, 4), arguing for a potential pathogenic role in the onset and progression of APS disease.

Despite recent advances in analytical methods, detection of aPT, and especially aPS/PT, has proven difficult to standardize because of the transient nature of the phospholipid-bound complex, which requires calcium ions, and the variable source/purity of phospholipids and antigen. Furthermore, even though it is assumed that aPS/PT interact with prothrombin, they may react against PS and other plasma proteins capable of interacting with PS (5). The aim of this study was to develop an ELISA assay to improve the identification of aPT in correlation with thrombosis.

MATERIALS AND METHODS

Protein Expression and Purification

All prothrombin variants used in this study were produced in Expi293 cells (Thermo Fisher Scientific, USA) in the presence of vitamin K and purified as described before (6). Briefly, the cDNA of human prothrombin (ProTWT, UniProtKB P00734) modified to include an epitope for the HPC4 antibody (7) at the C-terminus was cloned into a pDEST40 expression vector (Life Technologies, Inc.) and sequenced verified by Genewiz. Differently from ProTWT, ProTS525A-Biot carries 1) an extended C-terminal peptide sequence ^{1}GGGSGLNDIFEAQKIEWHE^{19} inserted after the HPC4-purification tag that can be specifically biotinylated *in vitro* by the biotin ligase enzyme (Avidity, USA) at the lysine residue 14 and 2) a single point mutation, namely the catalytic serine (S) 525 was substituted to alanine (A). Genetic engineering of the original cDNA was attained by PCR using the Quickchange Lighting kit (Agilent) and appropriate primers (Integrated DNA Technologies). Transfection of Expi293 cells was performed using Lipofectamine 3000 (ThermoFisher, USA) following manufacturers' instructions. Selection of stably expressing clones was initiated 24 hours after transfection using the antibiotic geneticin (G-418, GoldBio, USA). Prothrombin secreted in the media was purified in three sequential steps: immunoaffinity, ion-exchange chromatography and size exclusion chromatography, as detailed elsewhere (6). A key step for obtaining highly pure recombinant protein was to load the immunopurified material into a heparin column (HiTrap Heparin HP 1 ml, Cytiva) before passing the solution into a Q-column (HiTrap Q HP 1 ml, Cytiva). This is because prothrombin fragments generated during protein production, storage, and purification (namely thrombin and prethrombin-2), but not intact prothrombin, bind to heparin. *In vitro* biotinylation was performed using BirA500 biotin-protein ligase reaction kit (Avidity). Incorporation of biotin was verified using QuantTag Biotin Quantitation Kit (Vector Laboratories).

Biochemical and Biophysical Studies

Surface plasmon resonance (SPR), kinetic studies and single-molecule Förster resonance energy transfer (smFRET) experiments with ProT120/478 and ProT120/478/S525A-Biot labeled with AlexaFluor-555 and AlexaFluor-647 maleimide were performed as described before (6, 8–10). Briefly, confocal smFRET data were collected on freely diffusing molecules using a MicroTime 200 microscope (PicoQuant) and analyzed using the MatLab based software PAM (11) to generate FRET histograms from hundreds of events. smFRET-total internal reflection fluorescence (TIRF) experiments were carried out with an objective-type TIRF microscope, essentially as described elsewhere (12, 13). Briefly, glass coverslips coated with polyethylene glycol (PEG)-biotin (MicroSurfaces, USA) with additional coverage of 1% Tween-20 to prevent protein sticking to glass coverslips were treated with neutravidin followed by addition of ProT120/478/S525A-Biot. The sparsely covered surface resulting from immobilization of ProT120/478/S525A-Biot was then imaged at 32 frames per second using Andor iXon EMCCD camera in Tris 20 mM, pH 7.4, 145 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween-20 supplemented with Trolox (2 mM) and glucose scavenging system (0.8% w/v D-glucose, 1 mg/ml glucose oxidase, and 0.04 mg/ml catalase). Data were processed using IDL script available from the Ha lab at http://ha.med.jhmi.edu/resources/. Traces were extracted using MatLab, then fitted to a two state Hidden Markov Model (HMM) model using vbFRET (14).

Plasma Samples and IgGs

Collection of citrated plasma and purification of IgGs was described earlier (8). Briefly, venous blood was collected at the University of Padua in 3.8% sodium citrate (9:1) and centrifuged twice at 2000xg for 15 min at 4°C. Plasma was stored in 25 µL aliquots at -80°C ready for individual use. All the patients in this study participated in the TRAPS trial and gave their written informed consent to utilize their stored residual plasma in this study. APS was diagnosed according to the Sydney Criteria (15). Total IgG extracts were purified using Protein G spin columns (ThermoFisher, USA) followed by size exclusion chromatography to obtain pure IgG monomers. Each preparation was >98% pure as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The IgG concentration was determined by reading at 280 nm with a molar extinction coefficient of 1.00 M^{-1} cm^2.

ELISA Assays

Neutravidin (3100, Thermo Fisher Scientific, USA) was resuspended in water and its concentration determined by reading absorbance at 280 nm using a molar extinction coefficient of 1.66 M^{-1} cm^2. For the detection of aPT-Biot, one hundred microliters of a solution of neutravidin solubilized in 0.1M sodium bicarbonate pH 9.6 were added to a Nunc MAXISORP plate (MilliporeSigma, USA) and incubated overnight at 4°C. After washing three times with 200 µl/well of 20 mM Tris pH 7.4, 145 mM NaCl, 5 mM $CaCl_2$, Tween 20 0.02% (TBS-T), wells were blocked with 200 µl of 20 mM Tris pH 7.4, 145 mM NaCl, 5 mM CaCl2, 1% BSA (TBS-B) for 60 min at room temperature. One hundred microliters of ProTS525A-Biot or ProTS525A at the desired concentration were incubated for 60 min at room temperature before repeating the washing step. Next, one hundred microliters of plasma (1:100 v/v dilution) or IgGs prepared in TBS-B were added to each well and incubated

for 60 minutes at room temperature (18-22°C). Plates were washed 3 times TBS-T before adding 100 µl of 1:10,000 dilution of peroxidase conjugated anti-human IgG, γ-chain specific (A6029, MilliporeSigma, USA) for 60 minutes at room temperature. Finally, plates were washed three more times with TBS-T and then incubated with 100 µl of 1-Step 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate (34028, ThermoFisher, USA). After 30 minutes, the colorimetric reaction was quenched with 100 µl of TMB-stop solution. The optical density at 450 nm was recorded using a SPARK microplate reader (TECAN, Austria). Statistical analysis between the groups was performed using t-tests in Prism 9.0 (*** < 0.001). aPT-A and aPS/PT were detected using procedures published previously (8).

RESULTS

Recent studies from our laboratory have documented that aPS/PT, despite being heterogeneous, preferentially bind to the N-terminal portion of prothrombin, also known as fragment-1 (8). Because of this finding, we hypothesized that detection of aPT in correlation with thrombosis might be possible by using a prothrombin derivative that can be immobilized to ELISA plates at the desired density and with a defined orientation, i.e., pointing the N-terminal fragment-1 toward the solvent. To this goal, we created ProTS525A-Biot. ProTS525A-Biot is a catalytically inactive version of prothrombin, which is specifically biotinylated at the C-terminus and can therefore be immobilized onto neutravidin coated plates at the desired density and orientation. Since prothrombin spontaneously converts to thrombin over time, thus resulting in fragment-1 and prethrombin-1 (16), the S->A substitution was chosen to improve the long-term storage and stability of the protein at physiological pH and ionic strength concentrations.

ProTS525A-Biot was expressed in Expi293 cells in the presence of vitamin K. Size exclusion chromatography (SEC) after *in vitro* biotinylation reaction revealed that that the protein is highly pure and monomeric (**Figure 1A**). To confirm functional integrity and the presence of the S525A mutation, the conversion ProTS525A-Biot to thrombin by the prothrombinase complex was monitored by gel electrophoresis (**Figure 1B**) and by a chromogenic assay (6) (**Figure 1C**), respectively. As expected for a structurally intact yet inactive enzyme, ProTS525A-Biot was properly converted to thrombin by the prothrombinase complex (**Figure 1B**), but the resulting enzyme displayed no catalytic activity (**Figure 1C**). Given the importance of the N-terminal region of prothrombin in the context of aPS/PT binding, we performed additional SPR binding experiments using immobilized negatively charged liposomes to assess whether glutamic acids underwent proper γ-carboxylation. As shown in **Figure 1D**, binding of ProTS525A-Biot to liposomes was concentration dependent and saturable. The affinity constant calculated by plotting the response unit at equilibrium *versus* [ProTS525A-Biot] yielded a K_d=0.23 ± 0.10 µM (inset, **Figure 1D**). This value is close to previously reported values obtained for ProTWT under the same experimental conditions (8), confirming structural and functional integrity of the Gla-domain.

We have previously shown that prothrombin is a very dynamic molecule, adopting closed and open forms in solution (6, 10). To elucidate whether ProTS525A-Biot retains the same dynamic properties of ProTWT when free and bound to neutravidin, we performed confocal (**Figure 1E**) and TIRF-based smFRET experiments (**Figure 1F**), respectively. Based on previous studies, we labeled positions 120 in kringle-1 and 478 in the serine protease domain with the FRET pair AlexaFluor 555/647. We found that ProT120/478/S525A-Biot, like ProT120/478, interconverted between closed (high FRET) and open (low FRET) forms in solution (**Figure 1E**). Remarkably, a similar behavior was observed when ProT120/478/S525A-Biot was bound to neutravidin, documenting that the engineered tag, active site mutation and immobilization strategy do not affect the structural and dynamic properties of the antigen (**Figure 1F**).

After having established that ProTS525A-Biot retained identical structural and functional properties compared to ProTWT, we tested whether aPT could recognize ProTS525A-Biot bound to neutravidin plates (**Figure 2**). We initially used total IgG extracts that were purified from 5 APS patients with high titers of aPT-A and aPS/PT. The laboratory profile of these patients is shown in **Table 1**. Neutravidin at 1 µg/well was immobilized onto hydrophilic plates and, after extensive washing, 100 µl of ProTS525A-Biot at a concentration of 10 µg/ml was added. Non-biotinylated ProTS525A was used as a negative control. The results in **Figure 2B** show that aPT strongly reacted against ProTS525A-Biot. To differentiate these autoantibodies from aPT-A and aPS/PT, we called them aPT-Biot. Importantly, we observed no reactivity against neutravidin that was incubated with non-biotinylated ProTS525A. This result validates our design and documents no cross-reactivity of aPT against neutravidin. Intra and inter-assay variability were assessed by independently repeating the ELISA experiment three times, using IgG samples from 17 patients (**Table 2**). Intra-assay variability, calculated on triplicates of the same sample, was < 5%; inter-assay variability was, on average, 10% (min 3%, max 17%).

Given that a single molecule of neutravidin can theoretically bind up to 4 molecules of biotin, we next varied the density of ProTS525A-Biot (10, 1, 0.1, 0.01 µg/well) while keeping neutravidin constant (1 µg/well). Using P27 as a source of autoantibodies, we found that the signal increased hyperbolically reaching saturation at 1 µg/well of ProTS525A-Biot (**Figure 2C**). This datapoint defines the lowest concentration of ProTS525A-Biot to achieve maximum signal. Thus, the combination 1 µg/well of neutravidin and 1 µg/well of ProTS525A-Biot, unless otherwise specified, will be used from now on in our assays.

Given that the molecular weight of prothrombin and neutravidin are comparable (72 kDa *vs* 60 kDa), the results in **Figure 2C** suggest that clustering of prothrombin driven by multivalent neutravidin, not solely antigen density, stimulates antigen-antibody complex formation. To test this hypothesis, we systematically lowered the concentration of neutravidin, from 5

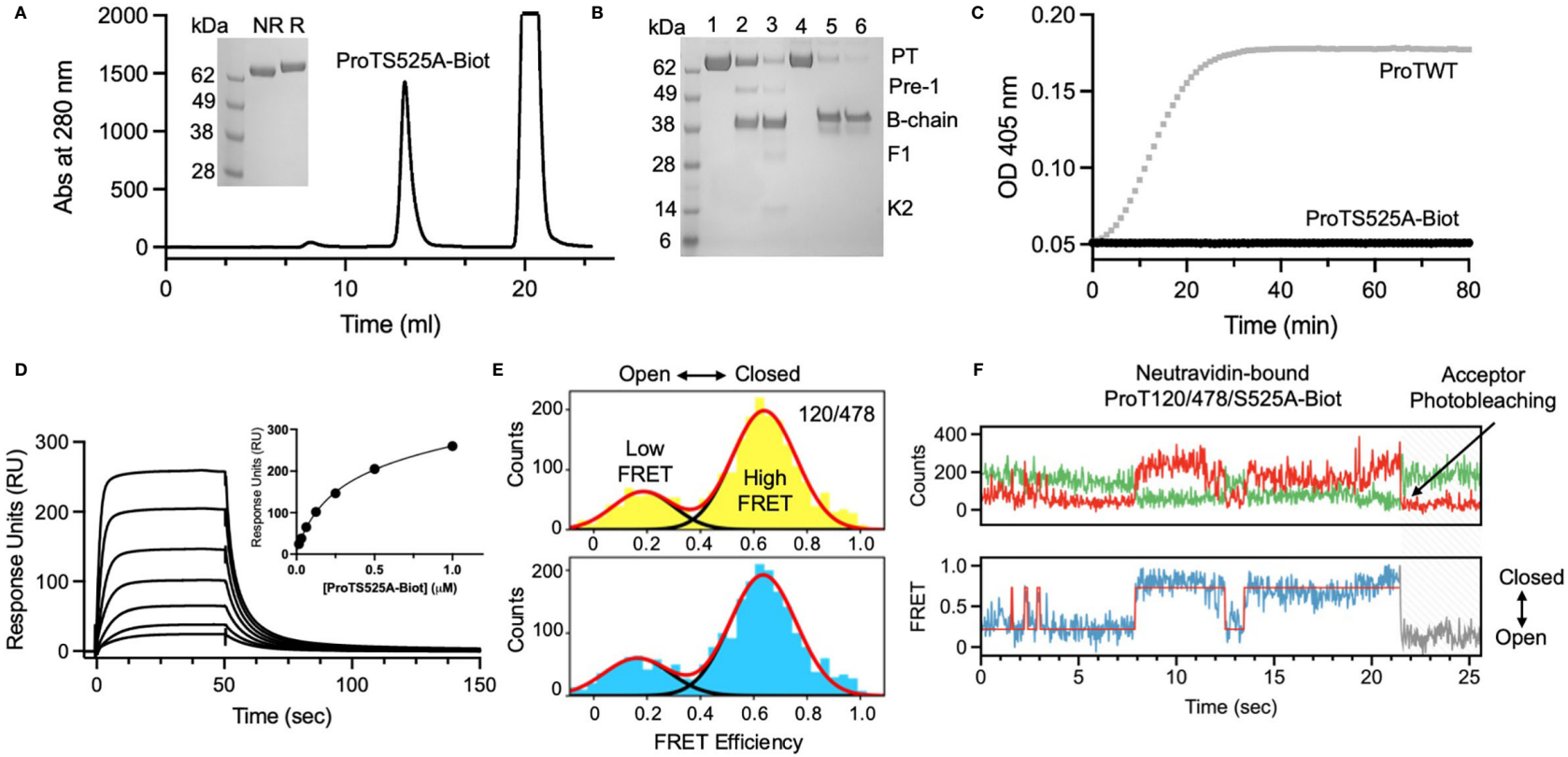

FIGURE 1 | Structural and functional characterization of ProTS525A-Biot. **(A)** Size exclusion chromatography (SEC) of ProTS525A-Biot on a Superdex S200 after biotinylation reaction. ProTS525A-Biot elutes at 13 ml. Excess of ATP needed for the biotinylation reaction is eluted with the void volume (20 ml). **(B)** Conversion of ProTWT (0.1 mg/ml, 1.4 μM, lanes 1,2 and 3) and ProTS525A-Biot (0.1 mg/ml, 1.4 μM, lanes 4,5 and 6) to thrombin by prothrombinase complex (fVa/fXa/POPC : POPS, 10 nM/0.4 nM/25 μM) in 150 mM NaCl, 20 mM Tris, 5 mM $CaCl_2$ monitored by gel electrophoresis. Following addition of prothrombinase complex (t=0, lanes 1 and 4), samples (40 μl) were quenched at 5 (lanes 2 and 5) and 10 minutes (lanes 3 and 6) with 10 μl of NuPAGE LDS buffer containing β-mercaptoethanol as the reducing agent and 20 mM EDTA. The samples were processed by NuPAGE Novex 4 –12% Bis-Tris protein gels run with MES buffer. Gels were stained with Coomassie Brilliant Blue R-250. Proteolytic fragments are indicated: prothrombin (PT), prethrombin-1 (Pre-1), B-chain, Fragment-1 (F1) and Kringle-2 (K2). Note how the autoproteolytic fragments Pre-1 and F1 are visible only in ProTWT but not in ProTS525A-Biot, which is catalytically inactive. Also note how the B-chain of ProTS525A-Biot has slower electrophoretic mobility compared to the B-chain of ProTWT due to the presence of the C-terminal biotinylated tag. **(C)** Conversion of ProTWT (25 nM, gray line) and ProTS525A-Biot (25 nM, black line) to thrombin by prothrombinase complex (fVa/fXa/POPC : POPS, 2.5nM/2.5pM/20μM) monitored using a continuous chromogenic assay (6). **(D)** Binding of ProTS525A-Biot (0.015-1 μM) to negatively charged liposomes (100 nm diameter extruded LUV made of POPC : POPS 80:20 molar ratio) monitored by SPR. **(E)** Confocal smFRET experiments of ProT120/478 (top) and ProT120/478/S525A-Biot (bottom) documenting similar distribution between closed (high FRET) and open (low FRET) forms in solution, with a ~80:20 ratio. **(F)** smFRET-TIRF obtained by immobilizing ProT120/478/S525A-Biot onto a neutravidin coated coverslips showing real-time dynamic exchange between closed (high FRET) and open (low FRET) conformations. Representative anticorrelated changes in AlexaFluor 555 (top panel, green) and AlexaFluor 647 (top panel, red) intensities and corresponding FRET changes (bottom panel). The red line in the bottom panel represents HMM fit to a 2-state model obtained using vbFRET (14). Also shown is a single photobleaching event of the acceptor dye occurring very rapidly at 21.4 sec, indicative of a single prothrombin molecule.

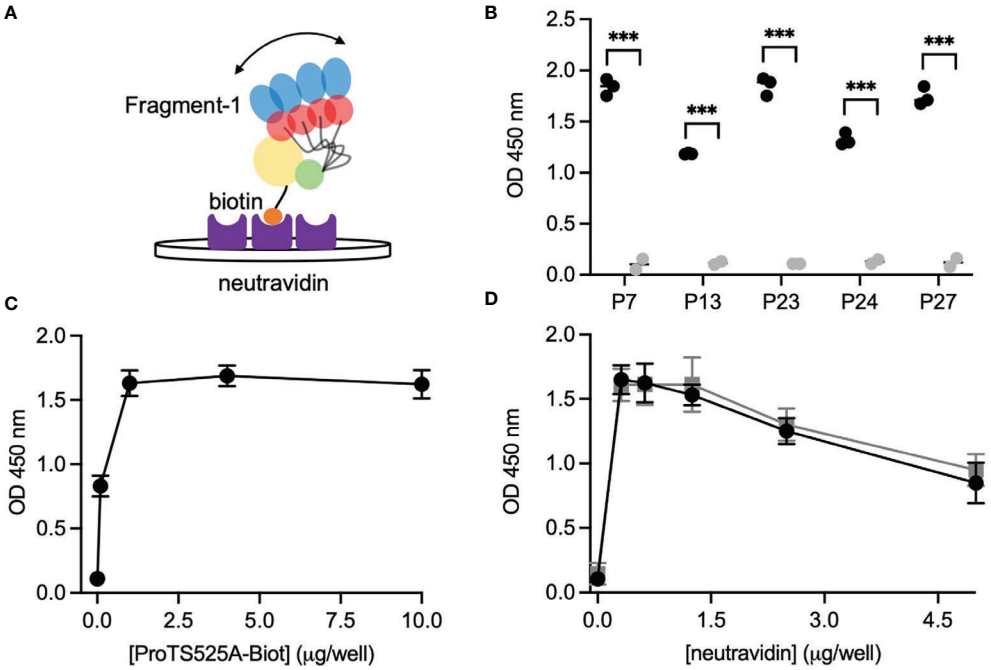

FIGURE 2 | Design and validation of the new ELISA assay. **(A)** Cartoon showing the immobilization scheme of ProTS525A-Biot onto neutravidin coated plates and mobility of fragment-1 swinging between closed and open states documented by smFRET. **(B)** Control experiments using biotinylated (black) and non-biotinylated (gray) ProTS525A. Statistical analysis between the groups was performed using t-tests in Prism 9.0 (*** < 0.001). Optimization experiments with different concentrations of ProTS525A-Biot **(C)** and neutravidin **(D)**. Total IgGs were purified as described before (8) and used at a concentration of 75 µg/ml. Representative results obtained with P27 are shown in **(C)**, whereas results with P7 (black) and P27 (gray) were chosen for **(D)**.

µg/well to 0.25 µg/well, while keeping ProTS525A-Biot constant (1 µg/well). We observed significantly lower signal at higher neutravidin concentration (**Figure 2D**). This effect is in line with our stated hypothesis, which predicts loss of binding at high concentrations of neutravidin due to antigen spreading. Finally, to test for stability, we stored plates with bound ProTS525A-Biot for up to 2 months at 4°C. No significant differences compared to freshly prepared plates was observed.

Given the promising results with total IgG extracts, aPT-Biot were researched in a cohort of 27 triple-positive APS patients available from our previous studies (8) (P01-P27) and 5 healthy controls (C01-C05) (**Table 1**), using 1:100 v/v diluted plasma and not total IgG extracts. This design was chosen to match more closely what happens in clinical laboratories. Importantly, since all the patients fulfill the definition of triple positive APS, they are considered at high risk of developing thrombosis (17, 18). Given a cutoff of 0.16 (OD 450 nm), defined as mean OD value for healthy controls +/- 3 STD, we found 24 out of 27 (88%) APS patients positive for aPT-Biot (**Figure 3A**). When compared to aPS/PT detected using the kit manufactured by INOVA (**Figure 3B**) and aPT-A (**Figure 3C**), aPT-Bio showed an excellent linear correlation with aPS/PT (R^2 = 0.85) but not with aPT-A (R^2 = 0.40). Interestingly similar considerations applied when we compared aPS/PT with aPT-A (**Figure 3D**), underscoring the similarity aPT-Biot and aPS/PT and confirming the difference between aPS/PT and aPT-A discovered in earlier studies (3).

DISCUSSION

Developing novel, robust, and easy to interpret immunogenic assays to detect autoantibodies in patients' plasma is a challenging yet essential task to achieve. In this study, we report the design and technical validation of a new ELISA assay for the detection of potentially pathogenic aPT in human plasma and provide initial evidence regarding its potential utility in APS patients.

One of the main problems faced by investigators when developing an ELISA assay is how to immobilize the antigen without perturbing its structural and functional properties, which are relevant for physiology and pathology. This problem needs to be considered even more carefully when antigens are inherently flexible and autoantibodies target conformational, and not linear epitopes, as is often the case for aPL (8, 19, 20). Here, we solved this issue by generating a recombinant version of prothrombin, which is site-specifically biotinylated and can be immobilized with proper orientation and desired density. Importantly, this construct retains its native structural, functional, and dynamic features in solution and when immobilized on a surface coated with neutravidin (**Figure 1**).

TABLE 1 | Laboratory profile of the 27 APS patients (P01-27) and 5 healthy controls (C01-C05) used in the study.

	aCL	aβ_2GPI	aPS/PT	aPT-A	aPT-Biot
P01	0.07	*0.14	1.06	0.25	0.76
P02	0.28	0.80	0.26	0.07	0.03
P03	1.01	2.32	3.48	1.78	2.60
P04	0.59	0.87	2.85	0.61	1.71
P05	0.41	0.65	0.22	0.05	0.34
P06	1.55	1.85	2.05	0.08	0.89
P07	0.28	0.13	3.23	2.19	2.67
P08	1.90	1.68	0.94	0.00	1.54
P09	0.93	0.40	3.34	2.08	2.34
P10	0.36	1.09	1.12	0.00	0.77
P11	0.36	0.71	0.50	0.00	0.32
P12	0.97	1.70	0.40	0.00	0.66
P13	1.55	2.43	2.04	1.00	1.28
P14	1.11	1.74	2.73	0.50	1.59
P15	1.31	1.35	0.36	0.01	0.19
P16	0.41	0.37	1.34	0.06	0.51
P17	1.26	2.14	2.04	0.06	0.98
P18	1.18	1.46	0.34	0.10	0.09
P19	0.36	0.87	1.70	0.00	0.48
P20	0.04	*0.11	1.76	0.18	1.10
P21	0.83	0.80	3.04	0.27	1.94
P22	1.56	1.66	0.17	0.00	0.04
P23	0.81	0.32	3.42	1.94	2.38
P24	0.79	1.57	2.67	0.31	1.22
P25	0.22	0.81	1.22	0.06	0.75
P26	1.85	2.38	2.07	0.18	0.66
P27	0.18	0.50	3.48	0.75	1.95
C01	0.05	0.01	0.11	0.05	0.01
C02	0.08	0.04	0.08	0.04	0.08
C03	0.04	0.04	0.08	0.80	0.04
C04	0.12	0.12	0.08	0.02	0.12
C05	0.10	0.10	0.08	0.02	0.14

Shown are values of OD 450 nm for each IgG autoantibody type.
aCL – anticardiolipin antibodies – Type: commercial (QUANTA Lite® ACA IgG III, Inova Diagnostics). Assay Description: Purified cardiolipin bound to the wells of a polystyrene microwell plate.
aβ_2GPI – anti-β_2GPI antibodies – Type: commercial (QUANTA Lite® ß2GP1 IgG, Inova Diagnostics). Assay Description: Purified β_2GPI bound to the wells of a polystyrene microwell plate.
aPS/PT – anti-phosphatidylserine/prothrombin antibodies – Type: commercial (QUANTA Lite® aPS/PT IgG, Inova Diagnostics). Assay Description: Plastic microwell plate wells are coated with purified PS/PT complex and then stabilized.
aPT-A – anti-prothrombin antibodies – Type: home-made (Maxisorp). Assay Description: Purified prothrombin bound to the wells of a polystyrene microwell plate.
aPT-Biot – anti-prothrombin antibodies – Type: home-made (Maxisorp). Assay Description: C-terminal biotinylated prothrombin bound to the neutravidin coated wells of a polystyrene microwell plate under conditions that preserve the antigen in its native state.
**P01 and P20 are positive for IgM aβ_2GPI.*

TABLE 2 | Inter-assay variability.

	E1	E2	E3	Ave	STD	%STD
P01	0.56	0.45	0.47	0.49	0.06	12%
P03	1.42	1.40	1.63	1.48	0.12	8%
P04	1.06	0.85	1.09	1.00	0.13	13%
P06	0.62	0.45	0.57	0.55	0.09	17%
P07	1.46	1.31	1.60	1.46	0.14	10%
P09	1.34	1.29	1.52	1.38	0.12	9%
P13	0.91	0.75	0.70	0.78	0.11	14%
P14	0.84	0.78	0.90	0.84	0.06	7%
P17	0.62	0.52	0.58	0.57	0.05	9%
P19	0.49	0.45	0.45	0.46	0.02	5%
P20	0.70	0.61	0.63	0.64	0.05	7%
P21	1.24	1.13	1.41	1.26	0.14	11%
P23	1.27	1.21	1.34	1.27	0.06	5%
P24	0.57	0.45	0.52	0.51	0.06	11%
P25	0.77	0.64	0.69	0.70	0.07	9%
P26	0.70	0.64	0.63	0.66	0.03	5%
P27	1.05	1.08	1.12	1.08	0.03	3%

Shown are values of OD 450 nm measured for each patient in three different experiments (E1, E2 and E3).
*IgG were used at a concentration of 50 µg/ml. ProTS525A-Biot was at 1 µg/well. ELISA experiments were repeated three times, on three different days. Each datapoint was run in triplicate. Ave, average; STD, standard deviation; % STD, Per Cent Standard deviation calculated as (STD/Ave*100).*

To our knowledge, this approach is novel and has never been applied before for the detection of aPT.

In addition to successfully detecting aPT in patients' plasma, a fascinating observation was that aPT-Biot correlated well with aPS/PT but not aPT-A. Since aPS/PT are an emerging biomarker of thrombosis in APS (4, 21) and are also found in other prothrombotic diseases such as COVID-19 (22, 23), this result is quite remarkable; it indicates that aPT-Biot may, too, find utility in the clinical practice. Rigorously designed prospective studies on a larger cohort of patients and matching controls will prove whether this idea has any merit.

Despite significant efforts and some progress, detection of aPL, and especially aPS/PT, still suffers from platform-dependent variability due to different protocols for immobilizing the antigens -which are often undisclosed due to the use of proprietary technologies- as well as the intrinsic time-dependent instability of the reagents that are immobilized onto the plastic plate (24, 25). In this context, detection of aPT-Biot provides several technical advantages compared to aPS/PT. First, provided availability of ProTS525A-Biot, this new ELISA format is very straightforward to prepare and perform, favoring transparency and reproducibility. Second, since the interaction between biotin and neutravidin is practically irreversible and calcium-independent, ELISA plates can be stored for months without loss of activity and, in principle, ELISA assays could be performed with plasma collected with a variety of anticoagulants, such as sodium citrate but also ethylenediaminetetraacetic acid (EDTA) – an interesting possibility whose validation, however, requires more experimental work. Third, since a subgroup of pathogenic aPL are known to recognize lipids and not proteins interacting with lipids (5), elimination of PS should eliminate confusions regarding which aPL are being detected.

From a biochemical standpoint, the fact that aPT-Biot correlates with aPS/PT is an intriguing finding since aPS/PT are believed to recognize cryptic epitopes resulting from the interaction of prothrombin with PS (3). While epitope mapping studies are necessary to compare similarities between these two groups of autoantibodies, because of our design and results in **Figure 3B**, aPT-Biot, like aPS/PT (8), may engage conformational epitopes that are, at least in part, contained in fragment-1. If this hypothesis is confirmed, the main role of PS could be to properly orient and concentrate the antigen on the lipid surface, thus facilitating autoantibody binding. This antigen orientation

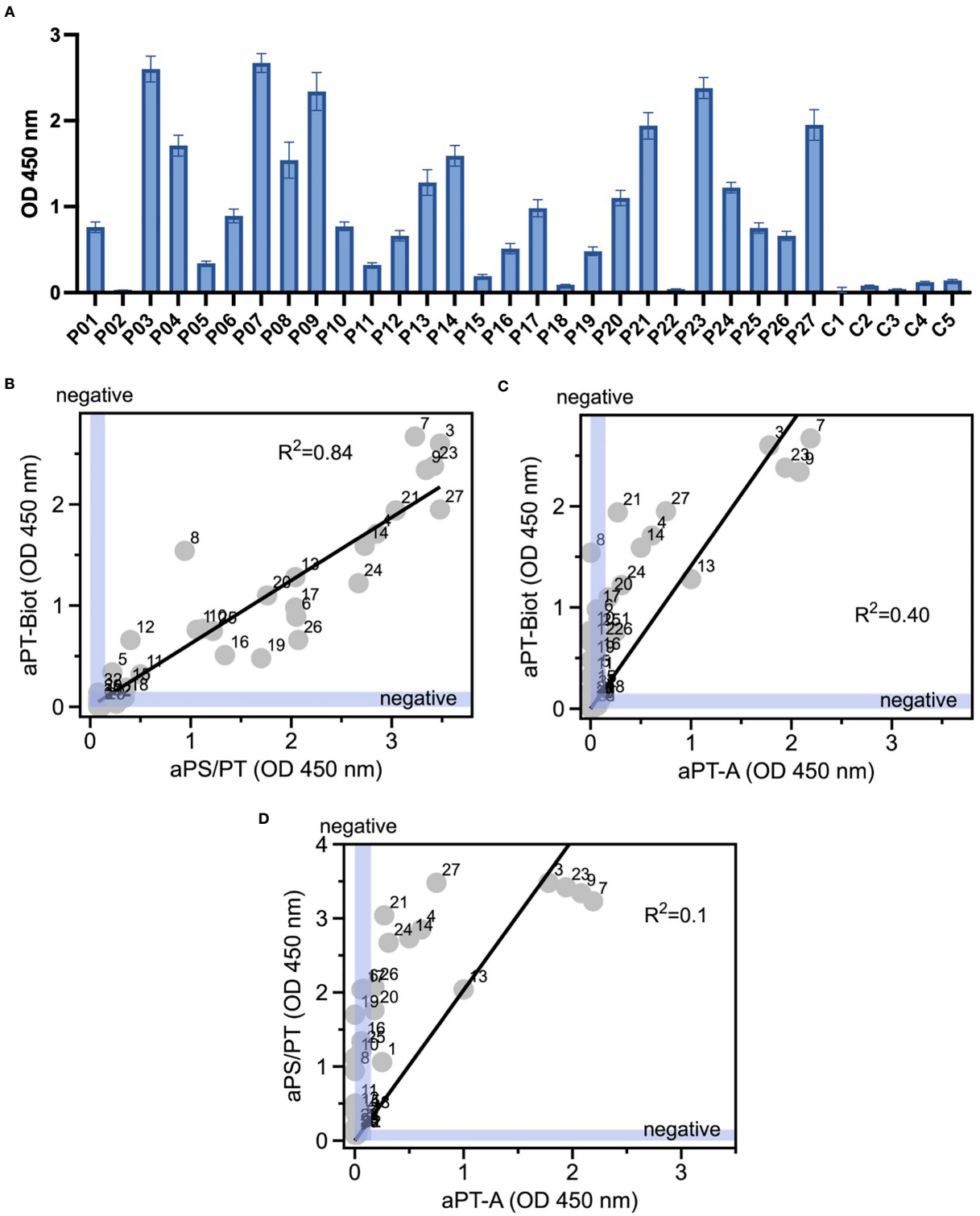

FIGURE 3 | Detection of aPT-Biot in patients' plasma and comparison with aPT-A and aPS/PT. **(A)** Detection of aPT-Biot in patients' plasma which was diluted 1:100 v/v in TBS-B. Correlations between **(B)** aPS/PT and aPT-Biot and **(C)** aPT-A and aPT-Biot. Negative intervals for x and y axes (0<OD<0.16) are highlighted in blue. Datasets were fit with a linear regression and the coefficient of determination (R^2) is shown in each plot. Each datapoint represents the average of three independent experiments run in duplicate. **(D)** Correlation between aPS/PT and aPT-A. Negative intervals for x and y axes are highlighted in blue. Datasets were fit with a linear regression and the coefficient of determination (R^2) is shown. Each datapoint represents the average of three independent experiments run in duplicate.

hypothesis could also help rationalize the remarkable difference between aPT-Biot and aPT-A, indicating that part of the prothrombin molecule recognized by aPT-Biot and aPS/PT is hidden or damaged when bound to hydrophilic plastic plates. However, it is also possible that aPT-Biot are different from aPS/PT and that, for example, aPS/PT might prefer epitopes that are unique in that they form when prothrombin is bound to PS, a situation that is reminiscent of recently discovered antiphospholipid antibodies targeting the endothelial protein C receptor in complex with lysobisphosphatidic acid (26).

In conclusion, we believe the new ELISA assay reported here represents an encouraging step towards a more specific detection method for aPT. Given the universality of the neutravidin-biotin system, it is also easy to envision how ProTS525A-Biot can be immobilized to other types of surfaces to enable highly reproducible and cost-effective detection of aPT in single and multiplex automated systems and to facilitate purification and characterization of aPT. Finally, a similar strategy could be applied to other antigens of aPL, such as β_2GPI, that, despite being primed for autoantibody binding in solution (20), is currently being absorbed onto plastic plates, a harsh method resulting in unpredictable effects on its native structure. In this context, it is worth noting that encouraging results towards a more personalized diagnosis of APS have been obtained using a fragment of β_2GPI, domain I (DI), which was either tagged at the N-terminus (27) or the C-terminus (28).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Padua (VP). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MC, VP, and NP designed, performed the research, and analyzed the data. NP wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Evaluation of the Diagnostic Value of Non-criteria Antibodies for Antiphospholipid Syndrome Patients in a Chinese Cohort

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Objective: Although specific anti-phospholipid antibodies (aPLs) have been used in the diagnosis of the antiphospholipid syndrome (APS) for years, new biomarkers are required to increase its diagnostic and risk-predictive power. This study aimed to explore the value of several non-criteria aPLs in a Chinese cohort.

Methods: A total of 312 subjects, namely, 100 patients diagnosed with primary APS, 51 with APS secondary to SLE, 71 with SLE, and 90 healthy controls, were recruited. Serum anticardiolipin (aCL) IgG/IgM/IgA, anti-β2-glycoprotein I (aβ2GPI) IgG/IgM/IgA, anti-phosphatidylserine/prothrombin antibodies (aPS/PT) IgG/IgM, and anti-annexin A5 antibodies (aAnxV) IgG/IgM were tested using ELISA kits.

Results: Of the total number of patients, 30.46% and 6.62% with APS were positive for aCL or aβ2GPI IgA, respectively, while 39.07% and 24.50% were positive for aAnxV or aPS/PT for at least one antibody (IgG or IgM). The addition test of aCL IgA and aAnxV IgM assists in identifying seronegative APS patients, and IgG aPS/PT was linked to stroke.

Conclusion: Detection of aCL IgA, aβ2GPI IgA, aAnxV IgG/M, and aPS/PT IgG/M as a biomarker provides additive value in APS diagnosis and would help in risk prediction for APS patients in medical practice.

Keywords: antiphospholipid syndrome, antiphospholipid antibodies, immunoglobulin A, anti-phosphatidylserine/prothrombin, anti-annexin V

INTRODUCTION

The antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by thrombosis and/or pregnancy morbidity with the persistent presence of medium or high titer of antiphospholipid antibodies (aPLs). The 2006 APS classification criteria (Sydney criteria) have been widely accepted in APS diagnosis, where at least one of the clinical criteria, as well as one of laboratory criteria including lupus anticoagulant (LA), high level of anti-cardiolipin (aCL), and

anti-β2 glycoprotein-I (aβ2GPI) immunoglobulin isotype G (IgG) or M (IgM), should be present (1). Despite its wide use in clinical practice, patients could remain persistently negative for criteria aPLs yet show typical APS clinical manifestations [defined as seronegative APS, SNAPS (2)], and a broader range of diagnostic biomarkers are required (3). Apart from standard criteria, other non-criteria clinical and laboratory features have been found associated with APS in numerous studies, such as heart valve disease, thrombocytopenia, neurological manifestations, anti-CL or anti-β2GpI IgA, anti-phosphatidylserine–prothrombin (aPS/PT) complex, and anti-annexin A5 antibodies (aAnxV) (4, 5). Besides APS diagnosis, evaluation of non-criteria aPLs could also contribute to prognosis and risk assessment for associated clinical manifestations (6).

More specifically, numerous studies have been conducted to investigate the diagnostic value of aCL/aβ2GpI IgA for APS, which received contradictory results (7). Nevertheless, testing of IgA had been recommended by guidelines when criterial aPLs remained negative (8). In addition, aAnxV and aPS/PT are receiving continuous attention in recent years. AnxV is a phospholipid-binding protein highly expressed in vascular endothelial cells. It could bind tightly to exposed anionic phospholipids and assemble into a shield, which may prevent phospholipid-dependent coagulation reaction (9, 10). In a systematic review, AnxV resistance has been observed and analyzed to have a higher prevalence in APS compared to disease controls (11) and has been reported to be linked to a hypercoagulable state as well as obstetric complications in APS patients (12, 13). Furthermore, its anticoagulant activity was reduced by plasmas of patients with APS and thromboembolism (14), and loss of maternal aAnxV increased the chance of placental platelet thrombosis and fetal loss (15). However, other studies found no significant association between thrombotic event or adverse pregnancy manifestations (16, 17).

Prothrombin is another phospholipid-binding protein that forms a complex and is often co-detected of antibodies together with phosphatidylserine (aPS/PT). An international multicenter study confirmed the contribution of aPS/PT IgG in APS diagnosis IgG (18). Concerning its relation with clinical features such as thrombotic events or obstetric complications, conflicting results had been shown and confirmation is still needed (19, 20). Nevertheless, numerous studies have indicated a strong correlation between aPS/PT and LA (21, 22). In addition, a higher level of aPS/PT was observed to be associated with high-risk "triple positive" patients (LA+, aCL IgG and/or IgM +, and aβ2GPI IgG and/or IgM+) (23), and may also add value to identification of SNAPS (3).

Study design, including detection method, patient stratification, population heterogeneity, and other factors, may lead to contradictory results in different studies. Regarding the Chinese population, a previous study indicated an increase of both IgG and IgM aAnxV in primary APS patients and APS associated with other diseases. Significant associations were also observed between IgG aANxV and thrombotic events (24). Additionally, assessment of the diagnostic performance of aPS/PT revealed a significant correlation between thrombotic events and pregnancy loss with IgG aPS/PT (25, 26), which was confirmed by a recent study (27). Concerning aCL/aβ2GpI IgA, a study recently conducted by us in a large Chinese population revealed little added diagnostic value (28). Few studies have explored all of the above non-criteria autoantibodies in the same patient groups, and their relations with more detailed clinical manifestations still need investigation. This study focused on evaluating the additive diagnostic value of aCL/aβ2GpI IgA, IgG and IgM for aANxV or aPS/PT to standard aPLs in a Chinese cohort. Correlation with clinical features including thrombotic events, obstetric complications, and microangiopathy was also explored.

PATIENTS AND METHODS

Patients Recruitment

This was a single-center, prospective cohort study conducted at Peking Union Medical College Hospital (PUMCH) from May 2017 to January 2020. A total of 152 consecutive APS outpatient cases were included in this study, of which 100 patients had been diagnosed with primary APS (PAPS group) and 51 with APS secondary to SLE (SAPS group). A total of 71 SLE patients (SLE group) and 90 healthy controls (HC group) were also included and matched with APS groups for gender and age. Diagnosis of APS was defined by clinicians according to the 2006 Sydney revised classification criteria. Upon diagnosis, sera samples were collected at the outpatient clinic and immediately profiled of aPL antibodies at the Key Laboratory of Department of Rheumatology, Peking Union Medical College Hospital (PUMCH). Besides aPL serology, history of clinical manifestations was recorded for PAPS, SAPS, and SLE groups, including thrombosis (arterial or venous), pregnancy morbidity, microangiography (i.e., thrombocytopenia, autoimmune hemolytic anemia), and history of adverse pregnancy. For the HC group, only aPL serology information was present. The study was approved by the ethics committee at PUMCH and fulfilled the ethical guidelines of the declaration of Helsinki. All subjects gave written informed consent.

Laboratory Tests

IgG, IgM, and IgA isotypes of aCL and aβ2GPI, IgG and IgM isotypes of aPS/PT and aAnxV were analyzed with AESKULISA® ELISA Test Kits provided by Aesku. Diagnostics GmbH & Co. KG (Wendelsheim, Germany). Cutoff value was defined as 18 U/ml as recommended by the manufacturer. Lupus anticoagulant was detected and evaluated at the Key Laboratory according to the ISTH recommendations measuring Dilute Russell viper venom time (dRVVT)/activated partial thromboplastin time (>1.20 as positive) (29). Diagnosis of SLE was based on the 1997 ACR criteria and confirmed by the 2019 EULAR/ACR criteria.

Statistical Analysis

Statistical analysis was performed using SPSS 26.0 or R (version 3.6.2). The χ^2 test or Fisher's exact test was used for comparison of categorical variables, and Wilcoxon test was used for continuous variables after normality was explored with the

Shapiro–Wilk test. Sensitivities, specificities, and accuracies in APS diagnosis were compared in the McNemar test. Youden Index, positive and negative predictive values (PPV and NPV), and odds ratio (OR) with 95% confidence interval (95% CI) were also shown. Receiver operating characteristic (ROC) curves of individual aPL as well as logistic regression analysis of aPLs profile were used to calculate the area under the curve (AUC), with 95% CI shown. Associations between aPL isotype positivity and clinical manifestation in patients with APS were explored and displayed in 95% CI. Two-tailed values of p less than 0.05 were considered statistically significant.

RESULTS

Patient Characteristics

Among 151 APS patients, there were 63 (63.0%) females for PAPS, 45 (88.2%) for SAPS, and the mean age for each was 36.3 and 32.9 years (**Table 1**). The mean age was 30.1 years in the SLE group, of which 61 (85.9%) were female, while the HC group had 41 (45.6%) females and a mean age of 43.4. A significant difference of female: male ratio was observed between PAPS and SAPS ($\chi^2 = 10.560$, $p = 0.001$). Clinical manifestations were recorded for both APS and SLE patients and were selectively shown. Thrombosis was most commonly present, with 80 (80.0%) for PAPS and 74.5% for SAPS, but not in the SLE group. Patients were recorded for history of arterial or venous thrombotic events, pregnancy morbidity, microangiopathy, history of adverse pregnancy, and LA. Of all the clinical manifestations, prevalence of adverse pregnancy history was significantly different between the PAPS and SAPS group ($\chi^2 = 3.922$, $p = 0.048$).

Predictive Power of aPLs in APS Diagnosis

The diagnostic power of aPLs positivity (>18 U/ml) was evaluated for sensitivity, specificity, accuracy, Youden Index, PPV, NPV, and ORs in APS diagnosis from HC group in **Table 2**. For IgA, the sensitivity and accuracy of the combination of aCL IgG, IgM, or IgA were significantly higher than that of aCL IgG or IgM ($p < 0.001$), while specificity was lower ($p = 0.031$). A similar result was observed for aCL or aB2GpI IgG or IgM or IgA compared to aCL or aB2GpI IgG or

TABLE 1 | Demographic and clinical variables of subjects (n = 312).

	APS (151)		SLE (71)	Healthy controls (90)
	Primary (100)	Secondary (51)		
Gender (female/male)	63/37	45/6	61/10	41/49
Mean age (years ± SD)	36.3 ± 12.1	32.9 ± 10.2	30.1 ± 8.2	43.4 ± 12.2
Clinical manifestations				
Thrombosis, *n* (%)	80 (80.0%)	38 (74.5%)	0	NA
Pregnancy morbidity, *n* (%)	33 (52.4%)	16 (35.6%)	0	NA
Thrombosis + pregnancy morbidity, *n* (%)	13 (20.6%)	3 (6.7%)	0	NA
LA, *n* (%)	73 (73.0%)	44 (86.3%)	17 (23.9%)	NA
History of arterial thrombosis, *n* (%)	43 (43.0%)	21 (41.2%)	0	NA
Stroke, *n* (%)	15 (15.0%)	4 (7.8%)	0	NA
Coronary heart disease, *n* (%)	5 (5.0%)	0	0	NA
Eye involvement, *n* (%)	3 (3.0%)	1 (2.0%)		
Lower limb artery occlusion, *n* (%)	1 (1.0%)	0	0	NA
History of venous thrombosis, *n* (%)	47 (47.0%)	24 (47.1%)	0	NA
Deep vein thrombosis, *n* (%)	19 (19.0%)	7 (13.7%)	0	NA
Pulmonary embolism, *n* (%)	19 (19.0%)	2 (3.9%)	0	NA
Upper limb vein thrombosis, *n* (%)	0	1 (2.0%)	0	NA
Renal vein thrombosis, *n* (%)	1 (1.0%)	0	0	NA
Portal vein thrombosis, *n* (%)	4 (4.0%)	1 (2.0%)	0	NA
Cerebral venous and sinus thrombosis, *n* (%)	3 (3.0%)	1 (2.0%)	0	NA
Central retinal venous occlusion, *n* (%)	1 (1.0%)	0	0	NA
Microangiopathy, *n* (%)	11 (11.0%)	13 (25.5%)	0	NA
Non-stroke CNS manifestations, *n* (%)	4 (4.0%)	4 (7.8%)	0	NA
Heart valve disease, *n* (%)	0	6 (11.8%)	0	NA
Antiphospholipid syndrome nephropathy, *n* (%)	6 (6.0%)	2 (3.8%)	0	NA
Hemolytic uremic syndrome, *n* (%)	1 (1.0%)	0	0	NA
Thrombotic microangiopathy, *n* (%)	0	1 (2.0%)	0	NA
Hematological disorder, *n* (%)	39 (39.0%)	33 (64.7%)	0	NA
Thrombocytopenia, *n* (%)	38 (38%)	*28 (54.9%)	21 (29.6%)	NA
Autoimmune hemolytic anemia, *n* (%)	1 (1.0%)	5 (9.8%)	0	NA
History of adverse pregnancy, *n* (%)	37 (58.7%)	20 (44.4%)	4 (5.6%)	NA
Early fetal loss (<10 weeks), *n* (%)	12 (19.0%)	8 (17.8%)	4 (5.6%)	NA
Late fetal loss (10–28 weeks), *n* (%)	19 (30.2%)	12 (26.7%)	0	NA
Recurrent fetal loss (>1 time), *n* (%)	11 (17.5%)	5 (11.1%)	2 (3.3%)	NA
Placental insufficiency, *n* (%)	14 (22.2%)	7 (15.6%)	0	NA

*$p = 0.048$, significantly different from primary APS; NA, not available.

TABLE 2 | The predictive value of different aPLs in APS diagnosis.

	Sensitivity (%)	Specificity (%)	Accuracy(%)	Youden Index	PPV (%)	NPV (%)	OR (95% CI)
aCL IgG	37.09	100.00	60.58	0.371	100.00	48.65	∞
aCL IgM	8.61	97.78	41.90	0.064	86.67	38.94	4.15 (0.91–18.81)
aCL IgG or IgM	41.06	97.78	62.24	0.389	96.88	49.72	30.65 (7.27–129.20)
aβ2GpI IgG	23.18	100.00	51.86	0.232	100.00	43.69	∞
aβ2GpI IgM	7.95	98.89	41.91	0.068	92.31	39.04	7.68 (0.98–60.12)
aβ2GpI IgG or IgM	29.14	98.89	55.19	0.28	97.78	45.41	36.60 (4.94–270.96)
aCL or aB2GpI IgG or IgM	43.05	97.78	63.48	0.408	97.01	50.57	33.26 (7.89–140.10)
aCL IgA	30.46	92.22	53.53	1.2268	86.79	44.15	5.19 (2.23–12.10)
aβ2GpI IgA	6.62	98.89	41.08	1.0551	90.91	38.70	6.31 (0.79–50.16)
aCL IgG or IgM or IgA	51.66	91.11	66.39	1.4277	90.70	52.90	10.95 (4.96–24.21)
aβ2GpI IgG or IgM or IgA	31.79	97.78	56.43	1.2957	96.00	46.07	20.51 (4.85–86.79)
aCL or aB2GpI IgG or IgM or IgA	53.64	91.11	67.63		91.01	53.95	11.86 (5.37–26.22)
P_1	**<0.001**	**0.031**	0.052				
P_2	0.125	1.000	0.375				
P_3	**<0.001**	**0.031**	0.052				
aPS/PT IgG	18.54	96.67	47.72	0.152	90.32	41.43	6.60 (1.95–22.40)
aPS/PT IgM	7.28	98.89	41.49	0.062	91.67	38.86	6.99 (0.89–55.10)
aPS/PT IgG or IgM	24.50	95.56	51.03	0.201	90.24	43.00	6.99 (2.40–20.32)
aCL, aB2GpI, or aPS/PT IgG or IgM	45.70	94.44	63.90	0.401	93.24	50.90	14.31 (5.49–37.25)
P_1'	**<0.001**	0.625	**<0.001**				
P_2'	0.167	0.375	0.064				
P_3'	0.125	0.250	1.000				
aAnxV IgG	30.46	100.00	56.43	0.305	100.00	46.15	∞
aAnxV IgM	16.56	96.67	46.47	0.133	89.29	40.85	5.75 (1.69–19.70)
aAnxV IgG or IgM	39.07	96.67	60.58	0.358	95.16	48.60	18.60 (5.62–61.53)
aCL, aβ2GpI, or aAnxV IgG or IgM	47.68	96.67	65.98	0.444	96.00	52.41	26.43 (8.01–87.26)
P_1''	0.648	1.000	0.503				
P_2''	**<0.001**	0.500	**0.007**				
P_3''	**0.016**	1.000	0.070				

PPV, positive predictive value; NPV, negative predictive value; OR, odds ratio; CI, confidence interval. p-values of sensitivity, specificity, and accuracy are calculated with McNemar test. P_1: Comparison of result of aCL IgG or IgM or IgA to aCL IgG or IgM; P_2: Comparison of result of aβ2GpI IgG or IgM or IgA to aβ2GpI IgG or IgM; P_3: Comparison of result of aCL or aβ2GpI IgG or IgM or IgA to aCL or aβ2GpI IgG or IgM; P_1': Comparison of result of aPS/PT IgG or IgM to aCL IgG or IgM; P_2': Comparison of result of aPS/PT IgG or IgM to aβ2GpI IgG or IgM; P_3': Comparison of result of aCL, aβ2GpI, or aPS/PT IgG or IgM to aCL or aβ2GpI IgG or IgM; P_1'': Comparison of result of aAnxV IgG or IgM to aCL IgG or IgM; P_2'': Comparison of result of aAnxV IgG or IgM to aβ2GpI IgG or IgM; P_3'': Comparison of result of aCL, aB2GpI, or aAnxV IgG or IgM to aCL or aβ2GpI IgG or IgM. Odds ratios (ORs) with 95% confidence intervals (CIs) are shown.

Bold values mean P < 0.05.

IgM. As for aAnxV, the sensitivity and accuracy of aAnxV IgG or IgM were significantly higher than that of aB2GpI IgG or IgM ($p < 0.001$). In addition, a combination of aCL, aβ2GpI, or aAnxV IgG or IgM had significantly higher sensitivity ($p = 0.016$) compared to that of aCL or aβ2GpI IgG or IgM.

As illustrated in **Figure 1**, ROC curves were applied to evaluate the predictive value of aPLs or their combined positivity. Among individual aPLs, aβ2GP1 IgG (0.915), aCL IgA (0.853), aCL IgM (0.767), and aAnxV IgG (0.728) had the largest AUC values. Adding IgA, aAnxV or aPS/PT IgG or IgM to aCL or aβ2GpI IgG or IgM would both increase AUC (0.927, 0.951, and 0.936, compared to 0.925).

Cross-Positivity Analysis for Four aPLs in APS Patients

Among 151 APS patients, cross-positivity of IgG, IgM, or IgA for aCL or aβ2GpI (a and b), as well as IgG or IgM for each of the four aPLs (c and d) were demonstrated with the Venn diagram in **Figure 2**. For patients positive for aCL, 16 were positive only for IgA. Concerning IgG isotype, aCL and aAnxV IgG were most often positive among APS patients. As for the IgM isotype, there were 12 (7.9%) patients who tested positive only for aAnxV, and 4 (2.6%) were positive only for aPS/PT.

For APS patients, 43 (28.5%) were positive for more than one non-criteria aPLs. Besides, the number of patients positive only for one of the five non-criteria aPLs was also calculated. A total of 11 patients positive only for aCL IgA, 9 for aAnxV IgG, 5 for aAnxV IgM, 3 for aPS/PT IgM, 1 for aPS/PT IgG, and 1 for aβ2GpI IgA were observed among these patients.

Distribution of Antiphospholipid Antibodies

The distribution of all criterial or non-criteria aPLs among different patient groups is shown in **Figure 3**. Levels of aPLs were calculated with (log(test value + 2)U/ml). The results of primary or secondary APS were compared to other groups. No significant difference was observed between primary and secondary APS, except for higher aCL IgM for PAPS ($p = 0.029$) and aβ2GpI IgA for SAPS ($p = 0.043$). Compared to HC, levels of IgG and IgA were significantly higher for four aPLs in both PAPS and SAPS group. However, IgM results varied for different aPLs.

Clinical Manifestations of Different aPLs in APS Patients

Correlations between different aPLs and LA or clinical manifestations are shown with odds ratios in **Table 3**. Presence of LA was significantly associated with IgG of aCL (ORs 9.0, 95% CI 2.6–

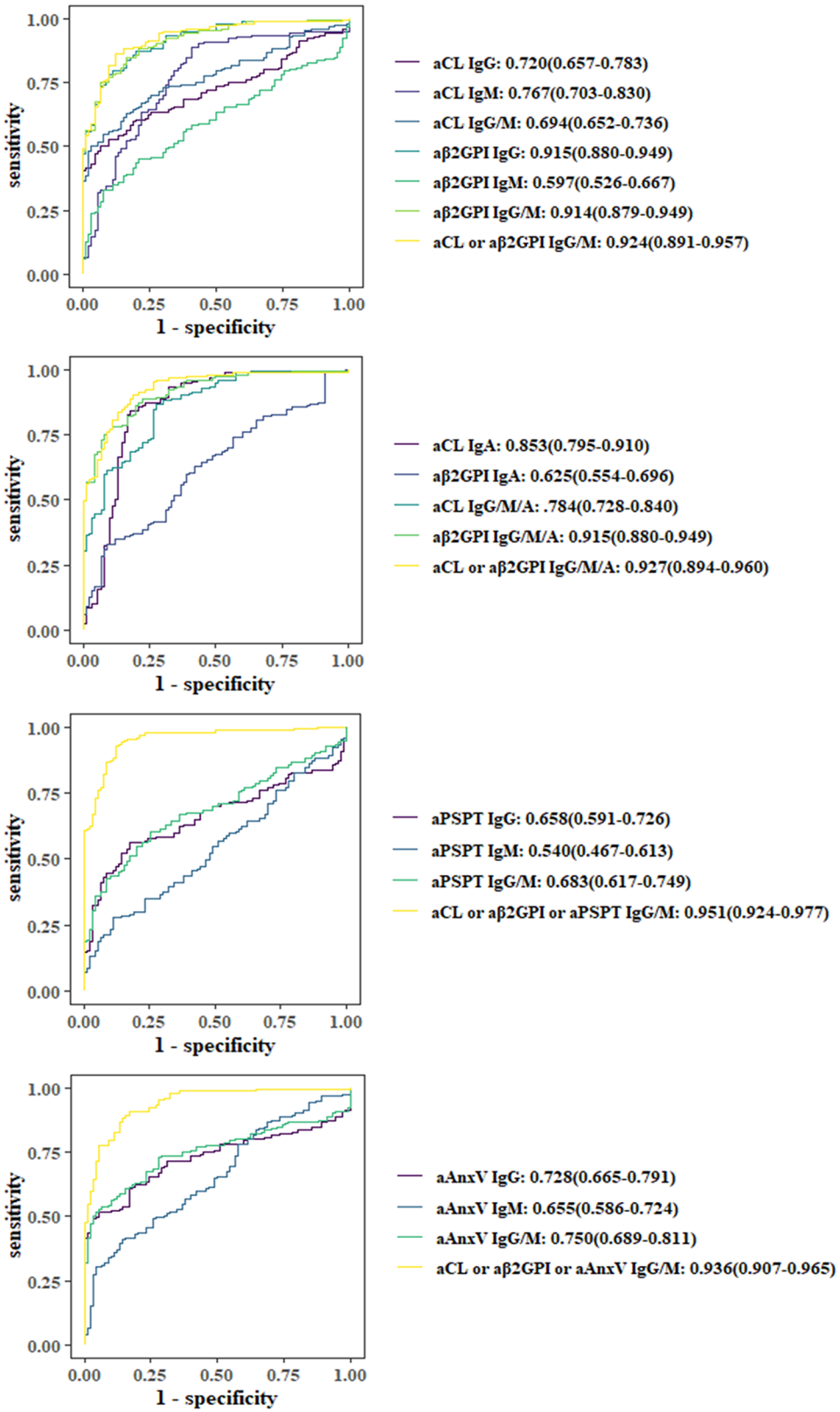

FIGURE 1 | Comparison of receiver operating characteristic (ROC) curves and area under the curve (AUC). ORs with 95% CIs are shown.

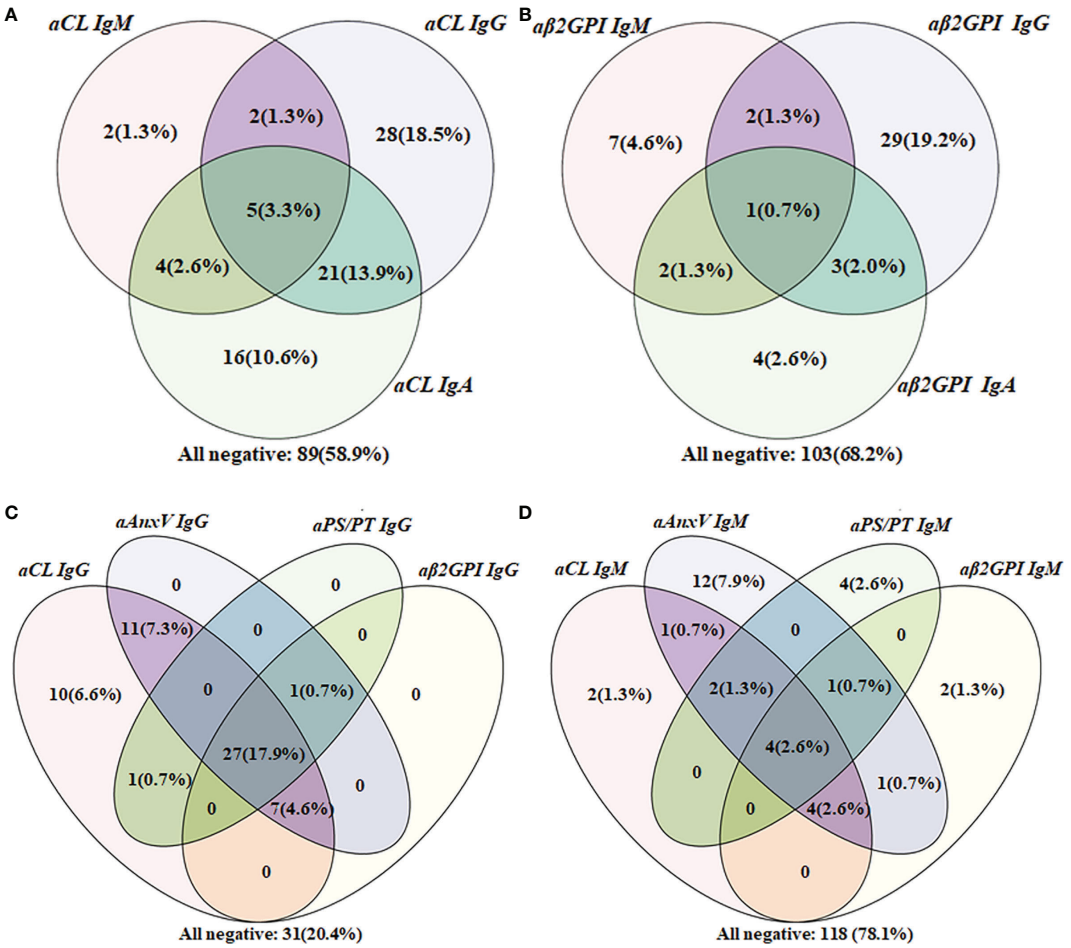

FIGURE 2 | Venn diagram of aPLs cross-positivity analysis in the APS group (n = 151). **(A)** Cross-positivity for aCL; **(B)** cross-positivity for aβ2GpI; **(C)** cross-positivity for IgG; **(D)** cross-positivity for IgM.

31.0), aβ2GPI (ORs 14.1, 95% CI 1.9–107.2), aPS/PT (ORs 4.7, 95% CI 1.1–21.2), and aAnxV (ORs 21.5, 95% CI 2.8–163.0). Among all vascular events, stroke was significantly associated with aβ2GPI IgG (ORs 2.8, 95% CI 1.0–7.7) as well as aPS/PT IgG (ORs 3.1, 95% CI 1.1–8.7). Additionally, aPS/PT IgM was reversely associated with pregnancy loss in women (ORs 0.6, 95% CI 0.5–0.7). LA positive was significantly related to thrombotic events (ORs 4.0, 95% CI 1.7–9.5), TP (ORs 4.1, 95% CI 1.7–10.2), and stroke (ORs 1.7, 95% CI 1.5–6.2).

DISCUSSION

APS is an autoimmune disease featuring thrombosis and/or pregnancy morbidity, which may lead to severe consequences. Detection of aCL and aβ2GPI as the golden standard in APS diagnosis is not satisfactory in the clinical scenario, and various potential aPLs have been extensively explored.

In this study, the diagnostic value of IgA for aCL or aβ2GPI and of IgG/IgM for aANxV or aPS/PT was evaluated in APS patients. In brief, 45.70% and 6.62% of patients with APS were positive for aCL or aβ2GPI IgA, respectively, while 30.46% and 24.50% were positive for aAnxV or aPS/PT for at least one antibody (IgG or IgM). Adding IgA to criterial aPLs could increase the sensitivity in APS diagnosis. Detection of aANxV or aPS/PT, especially aAnxV IgG, could add value to diagnosis. IgG of aANxV or aPS/PT was significantly associated with LA, and IgG aANxV was linked to stroke.

Analysis of the predictive power indicates that although aCL IgA had relatively low specificity, adding IgA to aCL IgG or IgM/aCL or aβ2GpI IgG or IgM test could increase test sensitivity (p < 0.001). The sensitivity (39.07% compared to 29.14%, p < 0.001) and accuracy (60.58% compared to 55.19%, p = 0.007) of aAnxV IgG or IgM were both significantly higher than that of aβ2GPI IgG or IgM. Moreover, combination of aCL, aβ2GpI, or aAnxV IgG or IgM had significantly higher sensitivity (47.7% compared to 43.0%, p = 0.016) than that of aCL or aβ2GpI IgG or IgM. Statistical results suggested that adding aAnxV IgG or IgM to aCL or aβ2GpI IgG or IgM would both increase diagnostic value besides criterial antibodies. Meanwhile, there was no significant decrease in specificity (96.67%).

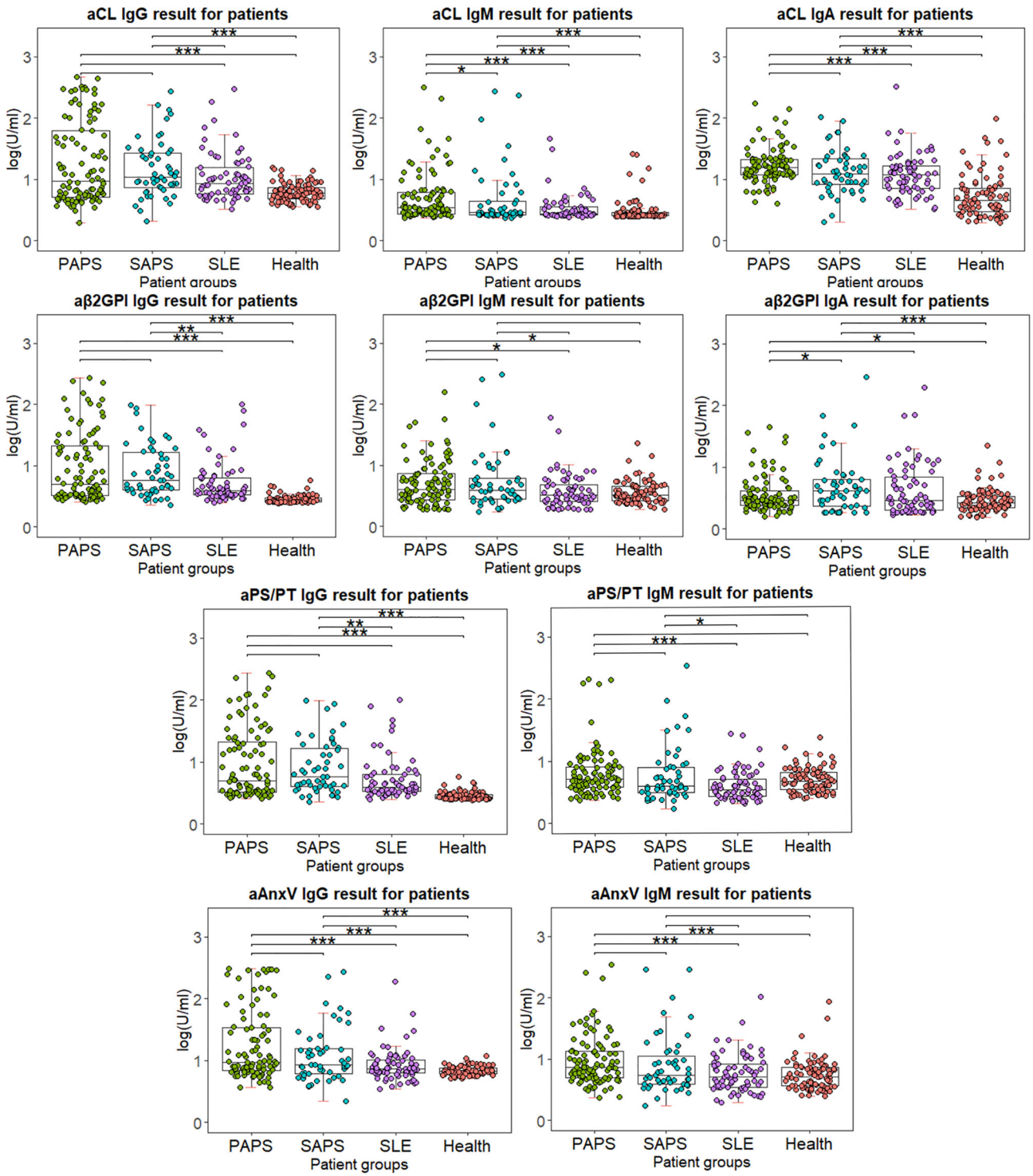

FIGURE 3 | Distribution of IgG and IgM for four antibodies among different patient groups. Test results are calculated using lg(test value + 2), in order for the value to be shown in positive number. Wilcox's test is conducted comparing primary or secondary APS results to other patient groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The result was further illustrated with ROC curves for each aPL and their combination. AUC of aCL IgA and aAnxV IgG ranked second and third (0.853 and 0.728) among individual aPLs. Statistical analysis showed that the addition of IgA to aCL IgG/IgM would significantly increase diagnostic power (AUC value 0.784 compared to 0.694). The addition of aAnxV or aPS/

TABLE 3 | Correlations between different aPLs and clinical manifestations among APS patients (n = 151).

	Thrombosis	Arterial thrombosis	Venous thrombosis	Pregnancy morbidity	Pregnancy loss	TP	Microangiopathy	Stroke	LA
aCL IgG	1.3 (0.6–3.1)	1.6 (0.8–3.2)	0.8 (0.4–1.6)	1.2 (0.6–2.7)	1.0 (0.5–2.3)	1.3 (0.7–2.6)	1.3 (0.6–2.4)	2.1 (0.8–5.5)	9.0* (2.6–31.0)
aCL IgM	0.6 (0.2–1.9)	0.8 (0.3–2.7)	0.7 (0.2–2.3)	1.0 (0.3–3.5)	0.5 (0.1–2.1)	1.6 (0.5–4.9)	0.5 (0.2–1.6)	1.3 (0.3–6.3)	3.9 (0.5–31.3)
aCL IgA	0.6 (0.2–1.2)	0.8 (0.4–1.5)	0.8 (0.4–1.5)	0.9 (0.4–1.9)	1.5 (0.7–3.2)	0.8 (0.4–1.5)	0.6 (0.3–1.2)	1.8 (0.7–4.8)	1.2 (0.5–2.5)
aβ2GPI IgG	1.7 (0.6–5.0)	1.6 (0.8–3.4)	1.0 (0.5–2.1)	1.1 (0.5–2.9)	0.9 (0.4–2.4)	1.3 (0.6–2.8)	1.4 (0.6–3.0)	2.8* (1.0–7.7)	14.1* (1.9–107.2)
aβ2GPI IgM	1.3 (0.3–6.5)	0.4 (0.1–1.6)	2.5 (0.7–8.6)	0.9 (0.2–4.2)	0.6 (0.1–3.0)	1.9 (0.6–6.3)	1.2 (0.4–4.1)	2.5 (0.6–10.5)	1.5 (0.3–7.5)
aβ2GPI IgA	1.1 (0.2–5.2)	0.9 (0.2–3.3)	1.8 (0.5–6.7)	1.0 (1.0–1.1)	0.7 (0.2–3.0)	0.8 (0.2–3.1)	0.6 (0.2–1.0)	0.8 (0.1–6.4)	2.9 (0.4–23.4)
aPS/PT IgG	1.3 (0.4–3.6)	1.7 (0.8–4.0)	0.7 (0.3–1.6)	1.3 (0.5–3.4)	0.9 (0.3–2.6)	1.1 (0.5–2.6)	1.4 (0.6–3.3)	3.1* (1.1–8.7)	4.7* (1.1–21.2)
aPS/PT IgM	1.2 (0.2–5.8)	0.8 (0.2–2.7)	2.1 (0.6–7.6)	0.4 (0.1–2.0)	0.6* (0.5–0.7)	1.6 (0.5–5.5)	0.3 (0.1–1.2)	0.7 (0.1–5.6)	3.2 (0.4–26.0)
aAnxV IgG	1.7 (0.7–4.3)	1.8 (0.9–3.6)	0.8 (0.4–1.7)	1.0 (0.4–2.3)	0.7 (0.3–1.7)	1.9 (0.9–3.7)	1.5 (0.8–3.1)	2.3 (0.9–6.1)	21.5* (2.8–163.0)
aAnxV IgM	1.1 (0.4–3.1)	1.3 (0.6–3.1)	0.7 (0.3–1.8)	1.0 (0.3–2.6)	0.9 (0.3–2.6)	1.8 (0.8–4.3)	1.4 (0.6–3.3)	2.7 (0.9–8.1)	2.5 (0.7–8.9)
LA	4.0* (1.7–9.5)	1.3 (0.6–2.9)	2.7* (1.2–6.1)	0.5 (0.2–1.1)	0.9 (0.4–2.2)	4.1* (1.7–10.2)	1.5 (0.7–3.2)	1.7* (1.5–6.2)	–

*Odds ratios (ORs) with 95% confidence intervals (CIs) are shown. *p < 0.05.*

PT to aCL/aβ2GpI IgG/IgM would also significantly increase diagnostic power (AUC value 0.951 compared to 0.924).

The Venn diagram indicated the additive value of new aPLs from another perspective. Positive only for IgA isotype could point out an extra number of patients for both aCL (16, 10.6%) and aβ2GPI (4, 2.6%). Additionally, the number of patients positive for aAnxV IgG, aAnxV IgM, and aPS/PT IgM outperformed those of aβ2GPI, indicating their importance in APS clinical diagnosis. The result suggested that additional tests for non-criteria aPLs may provide a unique value in the identification of SNAPS patients.

Besides predictive power, distribution, and comparison of aPLs among different patient groups were also examined. Between PAPS and SAPS, little significant difference was observed except for aCL IgM (p = 0.029) and aβ2GpI IgA (p = 0.043). Between PAPS and SLE, significantly higher titer of IgM aCL, IgA aCL, IgM aPS/PT, IgG AnxV, and IgM AnxV was observed (p < 0.001). As for SAPS and SLE, only IgM aPS/PT showed a significant difference (p = 0.015). The results implied that both criterial and non-criteria aPLs had difficulty in distinguishing APS from SLE or APS secondary to SLE. Indeed, baseline information suggested little difference between PAPS and SAPS patients in age and most clinical manifestations (**Table 1**). It had been estimated in previous studies that around 40% of patients with SLE have aPL, and APS may develop in up to 50%–70% of patients with both SLE and aPL (30). As all SLE patients were matched with APS groups for gender and age, we believed that this similarity in aPL results reflected the unique characteristic of aPL distribution in our population. Nevertheless, levels of IgG for four aPLs were significantly higher in both PAPS and SAPS group compared to HC, which suggested their diagnostic value.

Finally, the relationship between aPLs and related clinical manifestations was calculated. In this study, no significant association was found between aPLs with any thrombotic events, which was contradictory with results from some previous studies conducted in the Chinese population (24–26). Consistent with most previous findings, LA was strongly associated with thrombotic events including stroke (31). As the strongest predictor of APS-related features, LA was also a strong indicator of TP. Concerning obstetric complication, aPS/PT IgM was reversely associated with pregnancy loss in women (ORs 0.6, 95% CI 0.5–0.7), which also showed conflicting results (25, 32, 33). For aAnxV, similar to a previous study, no significant relationship was observed (24). The different results might be due to the detection system. ELISA was chosen in this study, and the cutoff value provided by the manufacturer (18 U/ml for all the aPLs) may not reflect real aPL distribution in local population. As illustrated in **Figure 2**, 31 patients were negative for all IgG, while as many as 118 patients were negative for all IgM. The cutoff value provided by the manufacturer might be overly strict and impact test sensitivity. It could be more suitable if the 99th percentile strategy was adopted first to identify cutoff points for each aPL. Regarding vascular manifestation, a significant relationship with aPLs (aβ2GPI IgG and aPS/PT IgG) was present for stroke. A previous review had estimated an aPL positivity of 17% in

patients with juvenile stroke (<50 years of age) (34), and incidence of stroke was up to 20% among APS patients in another cohort (35). Although detection of aPS/PT alone may have less diagnostic value, it would still be valuable in risk prediction for and prevention of adverse clinical events. Additionally, the relationship between aAnxV and aPS/PT IgG and LA was confirmed in our studies, and LA was found to be associated with IgG of all four aPLs.

This study has some limitations. Compared to similar studies, the sensitivity for some autoantibodies is not very high, which may influence the results of sequence comparison. Since different detection methods and manufacturers vary greatly in antibody measurement, contradictory results could arise (36). In the future, quantitative/semi-quantitative detection methods such as chemiluminescence analysis (CLIA) could be applied to reduce systemic detection error. In addition, both patients and healthy individuals involved in the study were relatively homogenous and may not reflect real-life condition. A larger sample size and inclusion of patients with a wider range of associated diseases or clinical features could further complement the study. Last but not least, the diagnostic power of other non-criteria aPLs such as aβ2GPI anti-domain I could also be explored with a similar procedure.

CONCLUSION

In conclusion, detection of aCL IgA, aβ2GPI IgA, aAnxV IgG/M, and aPS/PT IgG/M as a biomarker provides additive value in APS diagnosis, especially aCL IgA and aAnxV IgG. Detecting aCL IgA and aAnxV IgM assists in identifying seronegative APS patients. IgG of aANxV or aPS/PT was significantly associated with LA, and IgG aANxV was linked to stroke, which would assist in risk prediction for APS patients in medical practice.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Peking Union Medical College Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors were involved in the design of this study. CH, SL, ZX, HY, HJ, and JZ contributed to the collection of blood samples and other experimental procedures. YS and WQ were involved in data collection and pre-processing. CH and SL analyzed the data and wrote the manuscript. JZ, QW, XT, ML, and YZ contributed to the recruitment of patients and evaluation of clinical data. All authors contributed to the article and approved the submitted version.

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Sirolimus Monotherapy for Thrombocytopenia in Primary Antiphospholipid Syndrome: A Pilot Study From a Tertiary Referral Center

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Background: Thrombocytopenia (TP) is considered as a warning sign of high-risk antiphospholipid syndrome (APS) and sometimes a paradoxical sign of anti-thrombosis treatment. Currently, there is an extreme paucity of effective and safe drugs for long-term management of TP in primary APS patients; therefore, we explored the efficacy and safety of sirolimus monotherapy.

Methods: In this real-world study, we included 7 consecutive patients with primary APS who received sirolimus monotherapy for TP. Oral sirolimus was initiated at a dose of 1–2 mg once daily and then adjusted primarily based on clinical efficacy and tolerance, with consideration of the sirolimus trough concentration of ≤15 ng/ml.

Results: Of included patients, the median age was 58 years with a median disease course of 1.5 years and 4 patients were treatment-naïve. All patients completed 6 months of sirolimus therapy with a median follow-up of 6 months (range: 6–15). All patients received sirolimus monotherapy for TP during the entire follow-up, without any additional agents. Overall, the platelet count exhibited a substantially increasing trend after sirolimus administration during the first 6 months ($p < 0.001$) and stability later. Specifically, the median platelet count was significantly increased from 59×10^9/l before sirolimus to 90×10^9/l at month 1 ($p = 0.028$), 131×10^9/l at 3 months ($p = 0.028$), and 178×10^9/l at 6 months ($p = 0.018$). Overall and complete responses were respectively achieved in 6 (85.7%) and 5 (71.4%) patients at month 6. Importantly, overall response was achieved in all 4 treatment-naïve patients. Additionally, there were different extents of decline in the titers of antiphospholipid antibodies after sirolimus treatment. Regarding safety, only one patient experienced an elevated cholesterol level with recovery after atorvastatin treatment.

Conclusion: Sirolimus monotherapy confers good efficacy and tolerance for TP in primary APS patients and therefore may be considered as a first-line therapy.

Keywords: antiphospholipid syndrome, sirolimus, thrombocytopenia, real-world evidence, response

INTRODUCTION

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by the occurrence of vascular thrombosis or obstetrical complications in combination with the persistent presence of circulating antiphospholipid (aPL) antibodies. Thrombocytopenia (TP), one of non-criterion features of APS ranging from 15% to 53%, is currently considered to be of critical importance when managing APS patients (1, 2). A most recent investigation over a period of 38 years confirmed that the presence of TP, especially in persistent low-moderate conditions, was strongly associated with poor long-term survival of APS patients (3). However, there is an extreme paucity of effective, safe therapeutic drugs for the long-term management of APS-TP.

Sirolimus, as the mammalian target of rapamycin (mTOR) inhibitor, mainly inhibits cytokine receptor-dependent signal transduction and then blocks the activation of T cells, selectively increasing functional regulatory T cells. In a more recent study, sirolimus has been reported to be effective in connective tissue disease-related TP (CTD-TP) overall, but different types of CTD-TP appear to respond differently to sirolimus therapy (4). So far, there is no relevant study or case report on the efficacy of sirolimus for TP in APS patients, let alone sirolimus monotherapy. Therefore, we launched a pilot project to investigate the efficacy and safety of sirolimus monotherapy for TP in patients with primary APS.

MATERIALS AND METHODS

Study Design and Participants

This is a real-world study in Peking University First Hospital based on our dynamic cohort of APS from January 1, 2020 to December 31, 2021. Inclusion criteria were as follows: (1) with a definite or probable diagnosis (defined as aPL-positive but without classified manifestations) of primary APS, according to the 2006 Sydney criteria for APS (5), (2) aged ≥ 18 years, (3) presented with TP with PLT $<100 \times 10^9$/l (normal range 125–350 $\times$ 10^9/l), and (4) treated with sirolimus monotherapy for TP. Patients receiving sirolimus clinically for other conditions (e.g., APS nephropathy), or receiving glucocorticoid, immunosuppressive agents concomitantly or in the last 6 months before study entry were excluded. The study was approved by the Institutional Review Board of the Peking University First Hospital. The participants provided their informed consent to participate in this study.

Clinical Assessments and Data Collection

Baseline data of each eligible participant before initiation of sirolimus were collected, including demographics, symptom duration, APS-related manifestations, laboratory findings, and prior treatment details. After that, all patients were prospectively followed up monthly and then at least 3-monthly when platelet count reached at least 100×10^9/l and their condition was clinically stable. Additional follow-up was scheduled besides those at regular intervals if clinically necessary. The adjusted global APS score (aGAPSS) and damage index APS (DIAPS) were calculated, according to previous literature (6–8). Oral sirolimus was started at a dose of 1–2 mg once daily, and then doses were adjusted primarily based on clinical efficacy and tolerance, with consideration of sirolimus trough concentration of ≤15 ng/ml. The treatment decisions at each visit were made at the discretion of treating rheumatologists in clinical practice.

For both anticardiolipin antibody (aCL) and anti-β2-glycoprotein-I (β2GPI antibody detection, EUROIMMUN® ELISA IgG/IgM test kits were used in a EUROIMMUN® (Lübeck, Germany) fully automatic ELISA Analyzer I. Our local cutoff values for the aCL and anti-β2GPI antibody tests were set as validated manufacturer cutoffs (aCL IgG/IgM >12 GPL/MPL, anti-β2GPI IgG/IgM >20 GPL/MPL). The lupus anticoagulant (LA) test was performed with an ACL Top 700 Werfen® (L'Hospitalet de Llobregat, Spain) fully automatic coagulometer device with an integrated system of screening and confirmation steps. The APTT-based reagent is used for the antiphospholipid-dependent coagulation technique of the LA test. Confirmation tests were performed in aPTT with hexagonal-phase phospholipids (LA-SCT, Werfen) and in dRVVT with a phospholipid-rich dRVVT reagent (LA-DRVVT, Werfen). Our local cutoff value for the LAC test was set as the >99th percentile of distribution.

Outcomes and Assessments

In accordance with the immune thrombocytopenia (ITP) International Working Group Criteria (9), complete response was defined as a platelet count $\geq 100 \times 10^9$/l; partial response was defined as platelet count above 30×10^9/l, with at least doubling of the baseline platelet count. Overall response included complete response and partial response. The efficacy measures were the change in platelet count, overall response rate, and complete response rate after sirolimus therapy during the follow-up period. The safety outcomes included tolerance as assessed by the occurrence of common side effects.

Statistical Analysis

The trends of platelet count changes during the study period were analyzed using generalized estimating equations with an unstructured working correlation matrix and a robust estimation for the covariance matrix. The comparison of platelet count between baseline and month 1 or 3 was performed by using the non-parametric Wilcoxon signed rank-sum test. The cumulative probability of complete response and median time to complete response were calculated according to the Kaplan–Meier method. All the analyses were made using SPSS v.20.0. Microsoft Excel 2010, GraphPad Prism version 8.0, and R software were used to produce the graphs. The level of significance was set at a two-sided p value less than 0.05.

RESULTS

Patient Characteristics

In total, 7 consecutive patients with primary APS-TP were included. There were respectively 3 and 4 cases diagnosed as

having definite and probable primary APS, according to the 2006 Sydney classification criteria. Of 3 patients with definite primary APS, there were 2 patients with arterial thrombotic events and 1 patient with pregnancy complications. Baseline characteristics of 7 patients before sirolimus therapy are presented in **Table 1**.

At the start of sirolimus therapy, the median age of study participants was 58 (range: 19–61) years with a median disease course of 1.5 (range: 0.3–10.0) years. Regarding antiphospholipid antibody profiles, there were 5 out of 7 patients ever with triple positivity during the disease course. At the time of sirolimus therapy, there were 3 patients with triple positivity, 3 with double positivity, and 1 with single positivity. Among the 6 patients with LA positivity, none of them were receiving anticoagulant therapy when LA was tested. The median platelet count was 59×10^9/l (range: 33–98 $\times$ 10^9/l) before sirolimus therapy. Among 7 included patients, 4 patients were treatment-naïve and 3 patients were ever treated for TP, but with partial response in 2 patients and no response in 1 patient. In detail, glucocorticoid monotherapy was applied in 2 patients, and sequential therapies of prednisone, rituximab, vincristine, and thrombopoietin-receptor antagonist were adopted in the remaining 1 patient. The mean (standard deviation) aGAPSS and DIAPS for the included patients were 10.57 (5.29) and 0.57 (1.13), respectively.

In this pilot study, all participants were treated with sirolimus monotherapy for TP and regularly followed up till the final analysis. All the patients completed 6 months of follow-up with a median follow-up of 6 months (range: 6–15 months). In terms of sirolimus regimen, most participants (5/7, 71.4%) were treated with sirolimus at the dosage of 1 mg once daily without dosage adjustment during the entire period of follow-up. For patient 1, the sirolimus dose was escalated from 1 mg once daily at start to 2 mg once daily at 1 month due to insufficient efficacy and tapered to 1 mg once daily at 3 months and 0.5 mg once daily at 15 months due to favorable and stable efficacy, respectively. For patient 3, sirolimus was started from 2 mg once daily, escalated to 3 mg once daily at 2 months due to insufficient efficacy, and tapered to 1.5 mg qd at 7 months due to favorable and stable efficacy (**Table S1**). During the study observation, the trough concentration of sirolimus ranged from 2.6 to 12 ng/ml among the participants. Regarding concomitant therapy for APS, all 7 patients received hydroxychloroquine 200–400 mg/day. Additionally, 2 patients received aspirin and 1 patient received warfarin treatment (**Table S1**).

Change in Platelet Count

Figure 1 shows the platelet level before and after sirolimus treatment. Overall, the platelet count of included patients exhibited an increasing trend after administration of sirolimus ($p < 0.001$). In detail, the platelet count of 59×10^9/l (range: 33–98 $\times$ 10^9/l) before sirolimus therapy was significantly increased to 90×10^9/l (34–176 $\times$ 10^9/l) at the first month ($p = 0.028$), 131×10^9/l (56–241 $\times$ 10^9/l) at 3 months ($p = 0.028$), and 178×10^9/l (60–241 $\times$ 10^9/l) at 6 months ($p = 0.018$) after administration of sirolimus.

Correspondingly, overall response was achieved in 6 out of 7 patients (85.7%) at month 6, with 71% cumulative probability of overall response at month 4 and calculated median time to complete remission of 3.15 months during the treatment observation (**Figure 2**). Complete response was achieved in 5 out of 7 (71.4%) patients. For patient 6 who only achieved partial response but not complete response, the platelet count increased from 33×10^9/l at baseline to 77×10^9/l at month 6. Importantly, overall response was achieved in all of 4 treatment-naïve patients after receiving sirolimus. Of note, all these primary APS patients

TABLE 1 | Baseline characteristics of patients with primary APS-TP at enrolment.

Patients	Sex	Age at visit	TP duration (years)	APS types	Comorbidities	Clinical manifestations of APS	Lowest PLT ($*10^9$/L)	aGAPSS	DIAPS	aPL profile (ever)	Prior therapies for TP and response	PLT ($*10^9$/L) before sirolimus
1	F	61	5	Probable APS	Hypertension	TP	5	17	0	Triple positive	PSL with the highest dose of 30 mg/day (partial response)	59
2	F	58	0.2	Definite OAPS	Hyperthyroidism	Miscarriages, TP	66	4	0	Anti-β2-GPI positive	None	66
3	M	28	0.5	Definite TAPS	None	Stroke, TP, epilepsy	30	9	1	ACL/LA positive	PSL with highest dose of 100 mg/day, RTX, VCR, TPO-RA (partial response)	80
4	M	19	9	Probable APS	None	TP	40	5	0	Anti-β2-GP1/ LA-positive	PSL with highest dose of 150 mg/day (no response)	58
5	M	61	1.5	Definite TAPS	Hypertension Hyperlipidemia	Stoke, myocardial infarction, TP	98	17	3	Triple positive	None	98
6	F	58	0.5	Probable APS	Hyperthyroidism depression	TP, Alzheimer's disease	21	9	0	Triple positive	None	33
7	F	29	1.5	Probable APS	None	TP	50	13	0	Triple positive	None	50

TP, thrombocytopenia; APS, antiphospholipid syndrome; TAPS, thrombotic APS; OAPS, obstetrical APS; anti-β2-GPI, anti-β2-glycoprotein I; ACL, anticardiolipin antibodies; LA, lupus anticoagulant; PSL, prednisolone; MP, methylprednisolone; RTX, rituximab; VCR, vincristine; TPO-RA, thrombopoietin-receptor antagonist.

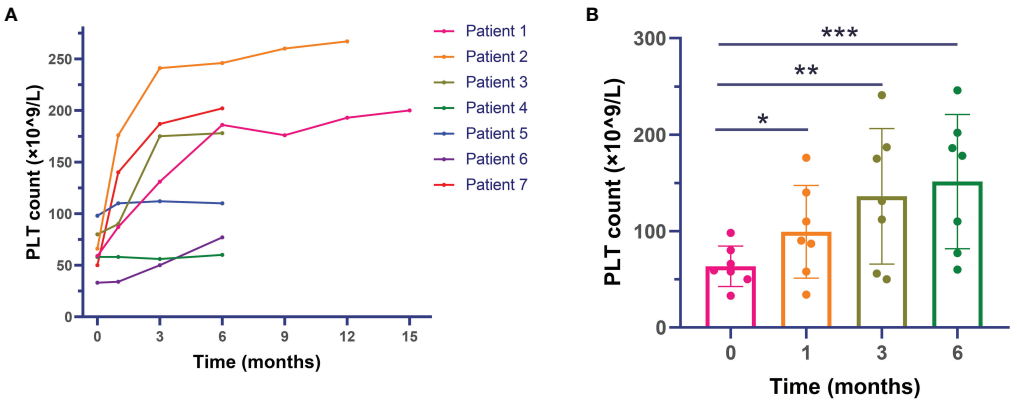

FIGURE 1 | Changes in platelet levels after sirolimus therapy in primary antiphospholipid syndrome patients with thrombocytopenia. **(A)** Platelet count of individual patients during the whole follow-up. **(B)** Median platelet count of included patients during the first 6 months. *: comparison between baseline and month 1 with $p < 0.05$; **: comparison between baseline and month 3 with $p < 0.05$; ***: comparison between baseline and month 6 with $p < 0.05$.

received sirolimus monotherapy for TP during the entire follow-up period, without any glucocorticoid, immunosuppressants, intravenous immunoglobulins, thrombopoietin-receptor agonist, etc.

Change in Antiphospholipid Antibody Profiles

There were 5 patients with repeated testing of aPL antibodies before and after sirolimus therapy. All these 5 patients did not receive anticoagulation therapy. There were different extents of decline in aPL antibodies after sirolimus exposure in all 5 patients as described in **Figure 3** with detailed information provided in **Table S2**. For 2 patients with positive ACL (both IgM and IgG), the titers of ACL antibodies were reduced after sirolimus therapy. For the titers of the anti-β2GPI IgM antibody, declines were observed after sirolimus treatment in all 4 patients with increased level before sirolimus. After sirolimus administration, a decrease in both LA-SCT and LA-DRVVT occurred.

Safety Measures

The sirolimus regimen was well tolerant. No patients discontinued sirolimus therapy due to safety concerns across the whole follow-up period. There was only a patient experiencing elevated cholesterol level, with low-density lipoprotein cholesterol from 3.14 mmol/l at baseline to 4.15 mmol/l at month 1 and resolve after atorvastatin treatment. No other clinically adverse effect was observed.

DISCUSSION

In this article, the good efficacy of sirolimus monotherapy for TP is reported for the first time in primary APS patients. After administration of sirolimus, platelet count was raised rapidly in the first 3 months and then increased steadily, yielding an overall response rate of 85.7% and complete response rate of 71.4%, respectively. During the whole follow-up period, no patients

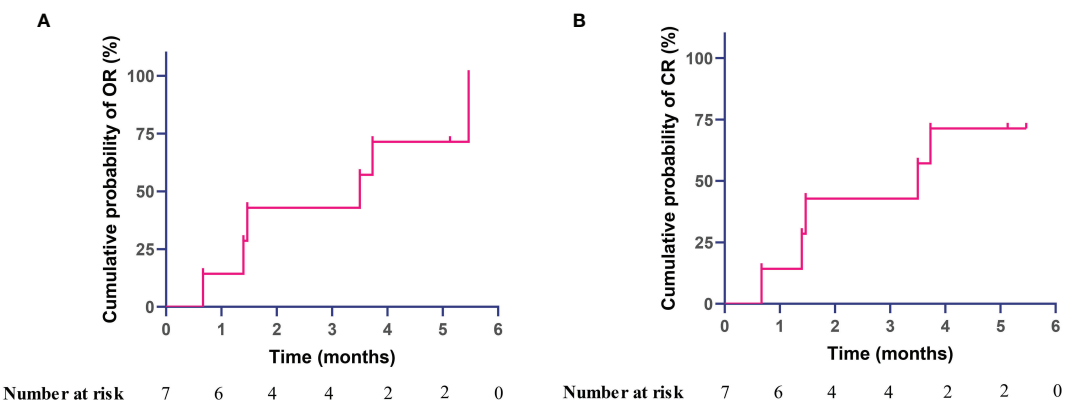

FIGURE 2 | Kaplan–Meier curve with cumulative probability of achieving response after sirolimus therapy in primary antiphospholipid syndrome patients with thrombocytopenia, **(A)** overall response, **(B)** complete response.

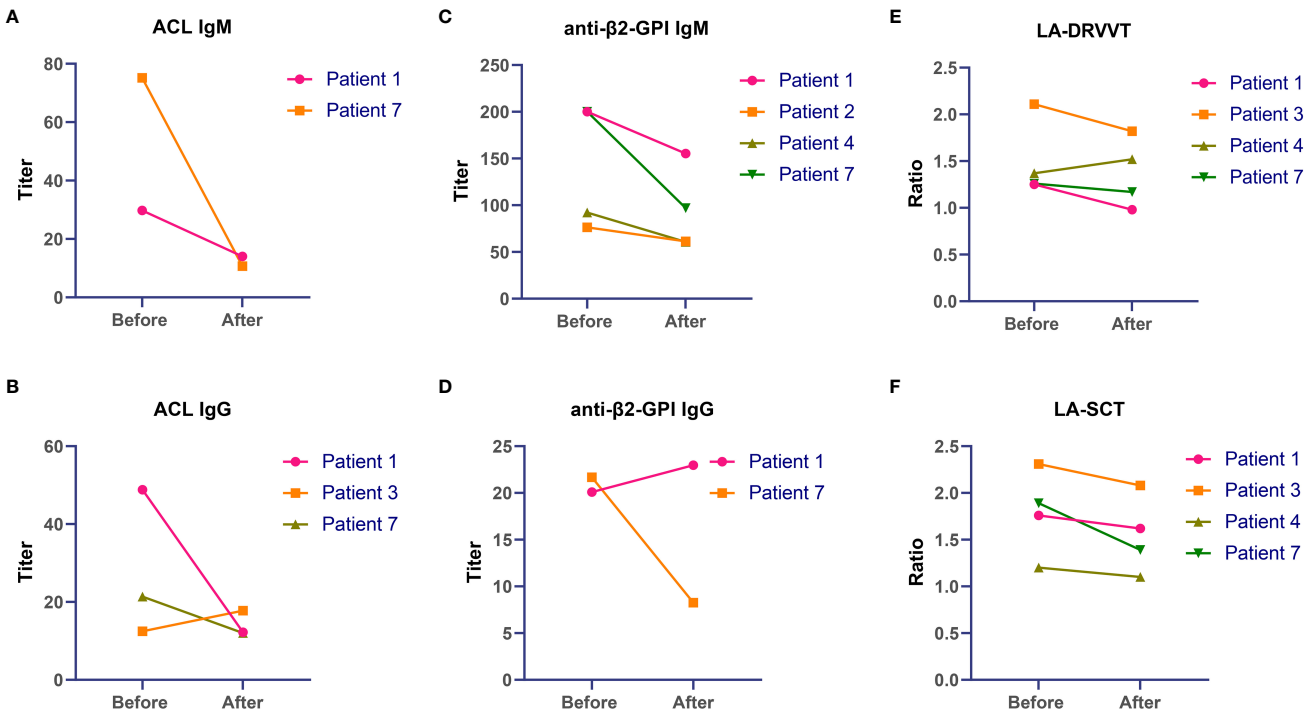

FIGURE 3 | Changes in antiphospholipid antibodies after sirolimus therapy in primary antiphospholipid syndrome patients with thrombocytopenia. **(A)** ACL IgM, **(B)** ACL IgG, **(C)** anti-β2-GPI IgM, **(D)** anti-β2-GPI IgG, **(E)** LA-DRVVT, **(F)** LA-SCT. ACL, anticardiolipin; anti-β2-GPI, anti-β2-glycoprotein I; LA, lupus anticoagulant.

discontinued sirolimus therapy due to safety concerns. These results suggest that sirolimus confers good clinical benefits for TP in primary APS with favorable safety and tolerability.

TP occurs in APS with a frequency ranging from 15% to 53%, without a completely clear cause and pathogenesis. TP in APS used to be considered as mild and benign, without the need for additional intervention in most cases. However, accumulating evidence has indicated that the presence of TP was significantly associated with poor long-term survival of APS patients (3). Active management with TP is therefore important to provide a better prognosis for APS patients. In general, glucocorticoid (i.e., prednisone 1–2 mg/kg/day) and high-dose intravenous immunoglobulins are used as the first-line treatment for severe TP in APS (10). However, both are inappropriate for long-term management in clinical practice with high frequency of adverse events and expensive cost. In American Society of Hematology 2019 guidelines for ITP, the task force recommends against a prolonged course of glucocorticoid treatment but is in favor of a short course (≤6 weeks) (11). For those with insufficient response or intolerance to glucocorticoid, immunosuppressants, such as azathioprine and cyclophosphamide, can be considered as second-line medications, but without sufficient supporting evidence for APS patients. Therefore, there is an urgent need to look for effective and well-tolerated treatments for TP in APS patients.

Currently, mTOR pathway activation has been demonstrated as one of critical mechanisms in the pathogenesis of APS (12, 13). Sirolimus, an inhibitor of the mTOR signaling pathway, has been reported for nephrology and cardiac microangiopathy associated with APS in a few studies (14–16). Regarding TP, several lines of investigation have shown that sirolimus treatment is associated with clinical benefits in pediatric ITP and CTD-TP (3, 17). However, the role of sirolimus in TP associated with APS has never been reported so far. In the present study, we found that platelet level was raised rapidly in the first 3 months and then increased steadily, with an overall response rate and complete response rate of 85.7% and 71.4% after sirolimus therapy, respectively. This may be attributed to several possible mechanisms. First, evidence from ITP revealed that impaired autophagy affects the differentiation of hematopoietic stem cells into megakaryocytes and differentiation of megakaryocytes into platelets (18). From this point of view, autophagy target treatment with sirolimus, as the autophagy inducer, has potential to elevate the platelet level in APS-TP *via* promoting platelet release from the bone marrow. Second, robust literatures have demonstrated that ITP is predominately a T cell disorder, characterized by abnormal T-cell responses, particularly defective activity of regulatory T cells. Therefore, novel therapeutics targeting T cells may be the most promising way to cure this disorder (19). Sirolimus mainly inhibits the activation and proliferation of T cells *via* inhibition cytokine receptor-dependent signal transduction but selectively expands functional regulatory T cells (20). Sirolimus hence increases the platelet level by facilitating the development of a regulatory loop

of T cells to alleviate the T cell-mediated destruction of platelet. Third, the presence of aPL antibodies has been thought to play important roles in TP associated with APS based on currently available data. The increased expression of platelet membrane glycoproteins and binding of the anti-β2GPI-β2GPI complex after aPL antibody stimulation lead to activation and aggregation of platelets (13). In a previous long-term observational study of 6 consecutive female primary APS patients with severe TP, B-cell depletion therapy with rituximab exhibited greatly sustained clinical efficacy (21). Sirolimus has been confirmed to block B-cell-activating factor-stimulated B-cell proliferation and survival by attenuating mTORC1/2-mediated intracellular free $Ca2^{+}$ elevations and suppressing the $Ca2^{+}$-CaMKII-dependent PTEN/Akt-Erk1/2 signaling pathway (22). Meanwhile, sirolimus is also found to significantly amplify regulatory B cells (23). Clinically, our latest study has comprehensively demonstrated the promising efficacy and good tolerability of sirolimus in patients with systemic lupus erythematosus (SLE) (24). Interestingly, different extents of decline in titers of aPL antibodies were observed after sirolimus exposure in the present study, suggesting that mTOR may play an important role in secretion of antibodies. The presence of aPL antibodies has been confirmed to directly mediate platelet aggregation, and sirolimus may therefore increase the level of platelet *via* inhibiting antibody-mediated causes of TP in APS. Taken together, sirolimus can elevate the platelet count in APS-TP by a variety of diverse mechanisms, including increase in platelet production and reduction in autoimmune-mediated destruction.

Of note, all included patients received the background therapy with hydroxychloroquine, which has been verified to have a multifaceted effect in primary APS, including the thromboprotective role as well as the potential to lowering aPL levels and increasing platelet count (25–27). In a most recent pilot open-label randomized prospective study, long-term hydroxychloroquine exposure was related to a decrease in some of aPL titers over an average 2.6-year follow-up (25). For TP therapy, adding hydroxychloroquine to a second-line treatment for TP has been confirmed as an attractive therapeutic approach in the treatment of children with ITP, particularly in the second line after failure of corticosteroids and/or IVIG (26). In adult patients, the recent study conducted by Khellaf et al. (27) similarly revealed that hydroxychloroquine seems to be a safe and efficacious second-line option for patients with SLE-ITP or ITP and high titer of antinuclear antibodies. At present, the potential to increase platelet count has not been directly confirmed in primary APS patients, which deserves further research in the future.

Experience from organ transplants indicated that the trough concentrations of sirolimus between 6 and 15 ng/ml are appropriate. This has been generalized to other diseases, including ITP and CTD-TP, but with insufficient supportive data. In fact, sirolimus 1–2 mg per day was commonly used for the autoimmune diseases, which is very different from the relatively high-dose regimens of sirolimus (mean daily dose 4.6 mg) in organ transplants (24, 28). In our study, over 80% of the participants attained overall response despite the trough concentrations of 6–15 ng/ml being reached in only 3 patients. This indicates that achieving sirolimus trough concentrations of 6–15 ng/ml may not be generalizable to autoimmune diseases. On the other hand, dose escalation or adjustment primarily based on clinical efficacy and tolerance is appropriate and should be recommended. In the study, sirolimus showed a good safety profile, which was consistent to previous studies (4, 17, 24). The good tolerability of sirolimus observed in our APS patients may be related to the relatively low serum concentration. Monitoring sirolimus trough concentration is still necessary.

There are several limitations that should be acknowledged. First, most of the included patients were with mild or moderate TP, rather than severe TP, because of safety considerations. In renal transplant recipients, the occurrence of TP in the sirolimus, cyclosporine, and prednisone regimen was significantly higher than that of the cyclosporine and prednisone regimen. We therefore selected patients with mild and moderate TP to preliminarily investigate the efficacy and safety of sirolimus for APS-TP in clinical practice. Second, a relative limitation of the study was absence of a control arm for comparison. In fact, to overcome this limitation, we were aided by the APS ACTION Executive Committee and Prof. Chengde Yang who governs the largest APS cohort in China, by providing the predesigned eligible control group treated by glucocorticoid monotherapy or no treatment for TP, although these data were finally failed to analyze due to the low number of eligible participants with necessary data. Other limitations include small sample size. This was just a pilot study, and more high-quality studies are clearly needed in the future to verify our results.

CONCLUSION

In conclusion, the pilot study showed good efficacy and tolerance of sirolimus monotherapy for TP in primary APS patients. Application of sirolimus as first-line therapy may be considered. A high-quality study with a larger number of patients is needed in the future to verify our results.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Peking University First Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZZ conceived and coordinated the study, was responsible for the management of some patients, and critically revised the manuscript. WX had full access to all the data collection, analysis, and interpretation and drafted the manuscript. LJ contributed to the study design, follow-up of patients and process of data collection. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.857424/full#supplementary-material

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PEGylated Domain I of Beta-2-Glycoprotein I Inhibits Thrombosis in a Chronic Mouse Model of the Antiphospholipid Syndrome

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Antiphospholipid syndrome (APS) is an autoimmune disorder in which autoantibodies cause clinical effects of vascular thrombosis and pregnancy morbidity. The only evidence-based treatments are anticoagulant medications such as warfarin and heparin. These medications have a number of disadvantages, notably risk of haemorrhage. Therefore, there is a pressing need to develop new, more focused treatments that target the actual pathogenic disease process in APS. The pathogenic antibodies exert their effects by interacting with phospholipid-binding proteins, of which the most important is beta-2-glycoprotein I. This protein has five domains, of which the N-terminal Domain I (DI) is the main site for binding of pathogenic autoantibodies. We previously demonstrated bacterial expression of human DI and showed that this product could inhibit the ability of IgG from patients with APS (APS-IgG) to promote thrombosis in a mouse model. Since DI is a small 7kDa protein, its serum half-life would be too short to be therapeutically useful. We therefore used site-specific chemical addition of polyethylene glycol (PEG) to produce a larger variant of DI (PEG-DI) and showed that PEG-DI was equally effective as the non-PEGylated DI in inhibiting thrombosis caused by passive transfer of APS-IgG in mice. In this paper, we have used a mouse model that reflects human APS much more closely than the passive transfer of APS-IgG. In this model, the mice are immunized with human beta-2-glycoprotein I and develop endogenous anti-beta-2-glycoprotein I antibodies. When submitted to a pinch stimulus at the femoral vein, these mice develop clots. Our results show that PEG-DI inhibits production of thromboses in this model and also reduces expression of tissue factor in the aortas of the mice. No toxicity was seen in mice that received PEG-DI. Therefore, these results provide further evidence supporting possible efficacy of PEG-DI as a potential treatment for APS.

Keywords: antiphospholipid syndrome, beta-2-glycoprotein I, PEGylation, domain I, thrombosis

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune disease in which autoantibodies cause clinical features of arterial or venous thrombosis or pregnancy morbidity. The pathogenic antibodies in APS are generally termed antiphospholipid antibodies (aPL) although they generally bind phospholipid-protein complexes. APS has a population prevalence of approximately 1 in 2000 (1) and can be diagnosed where a patient has at least one clinical feature (thrombosis or pregnancy morbidity) together with persistent positivity in at least one of the three serological assays for aPL that were cited in the most recent classification criteria for APS (2) and are in routine clinical use. These assays are the anti-cardiolipin (aCL) ELISA, the lupus anticoagulant assay and the anti-beta-2-glycoprotein I ELISA. Beta-2-glycoprotein I is present in a concentration of 200mcg/ml in human serum and has a wide range of biological functions including roles in both the complement and coagulation cascades (3). Pathogenic antibodies in APS primarily bind the N-terminal domain (Domain I) of beta-2-glycoprotein I (4, 5). This leads to the formation of trimeric complexes comprising one antibody molecule and two beta-2-glycoprotein I molecules. These complexes interact with anionic phospholipids and membrane receptors in the surface membranes of target cells such as monocytes, endothelial cells and platelets. The interaction stimulates change in cellular behavior such as release of tissue factor (TF), thus leading to the clinical features of the disease (6).

Current therapeutic options for APS are limited. The only evidence-based treatment to prevent recurrent thrombosis is long-term anticoagulation (7–9). This is usually achieved by prescribing vitamin K antagonists such as warfarin with disadvantages including the need for regular monitoring of blood tests and risk of haemorrhage. Although it was hoped that the introduction of direct acting oral anticoagulants such as rivaroxaban would reduce reliance on warfarin in patients with APS (10), recent trials have not favored this option (11, 12). In patients who test positive for aPL, but who have not yet suffered thrombosis, there is no strong evidence base for any treatment to protect against the first thrombotic event, though aspirin can be used in patients with a particularly high risk profile (9, 13).

It is therefore important to develop new forms of therapy for APS (12) which, rather than causing non-specific anticoagulation, block the pathogenesis of the syndrome in a more specific manner. One possibility is to block binding of pathogenic aPL to Domain I of beta-2-glycoprotein I (DI). Previously we developed a bacterial expression system for DI (14) and showed that the recombinant protein produced was able to block thrombosis induced by passive administration of IgG purified from serum of patients with APS (APS-IgG) in a mouse model (15).

DI is a small 7kDa peptide and therefore unsuitable for therapeutic use because its serum half-life would be very short. A number of techniques have been used to increase the size of such molecules to improve their therapeutic potential. These include addition of the immunoglobulin Fc region as in the anti-tumor necrosis drug etanercept and chemical addition of a polyethylene glycol (PEG) group. The latter process is called PEGylation and has been used in a number of drugs in current therapeutic use, notably pegloticase used in gout and certolizumab pegol used in rheumatoid arthritis [reviewed in (16)]. The size and method of attachment of the PEG group can both be modified. Larger PEG groups are beneficial in terms of increasing half-life and reducing immunogenicity but could potentially reduce biological activity by blocking interaction of the peptide with its biological ligand (17, 18). Whereas non-specific PEGylation of a peptide leads to a range of possible products with variable activity, site-specific PEGylation on disulfide bonds allows predictable and reproducible properties of the PEGylated product (19). However, development of anti-PEG antibodies can be a problem and has been described particularly for pegloticase, where it reduces the efficacy of the drug (20).

Since DI carries two disulfide bonds, it is suitable for site-specific PEGylation. In a previous issue of *Frontiers in Immunology*, we described site-specific PEGylation of DI and the purification and characterization of the PEGylated product (21). We showed that the PEG-DI obtained was able to inhibit binding of APS-IgG to whole beta-2-glycoprotein I, to inhibit the effect of APS-IgG on clotting of human blood and to inhibit the ability of passively transferred APS-IgG to cause thrombosis in a mouse model (21).

This passive transfer model, in which a relatively large amount of extraneous IgG is administered to a mouse acutely, is an imperfect model of APS because the antibodies are present persistently in patients with that disease. Therefore, Pierangeli and colleagues developed an alternative model in which mice are immunized with human beta-2-glycoprotein I and develop anti-beta-2-glycoprotein I antibodies (22). When the mice were submitted to a traumatic stimulus at the femoral vein, the size of the thrombus produced was significantly larger than in control mice immunized with human serum albumin.

In this paper, we investigate the inhibitory effects of PEG-DI on thrombosis in this chronic mouse model of APS.

METHODS

Production of PEG-DI

Bacterial expression and PEGylation of DI were described fully in our previous paper in Frontiers in Immunology (21). In brief, *E. coli* BL21* cells are transfected with the recombinant DI plasmid and expression of DI is achieved by addition of 1 mM IPTG followed by incubation with shaking overnight at 20°C. The PEG-DI originally collects in inclusion bodies, which are solubilized in a chaotropic buffer by bacterial lysis, sonication and centrifugation followed by grinding using a mortar and pestle. The expressed protein carries an N-terminal hexahistidine tag such that it can be purified on a nickel column. The purified protein is re-folded in 0.6 M arginine buffer with a cysteine-

Abbreviations: APS, Antiphospholipid syndrome; PEG, Polyethylene glycol; DI, Domain I of beta-2-glycoprotein I; TF, Tissue factor; CL, cardiolipin; β2GPI, beta-2-glycoprotein I.

cystine buffer (pH 8.5) and dialysed against 20 mM Tris, 0.1 M NaCl, pH 8. Protein is again purified post-folding using a nickel column and dialysed against phosphate buffered saline (PBS).

Protein was reduced at a concentration of 0.4 mg/ml in 2 M arginine, 20 mM sodium phosphate (NaPO4, 0.1 M NaCl), 40 mM EDTA at pH 8.0 with 0.1 M DTT for 1 h at 20°C. This process was followed by removal of the reductant and buffer exchange on a PD-10 column to an identical buffer with 25 mM arginine rather than 2 M. PEGylation reagent was added (1:0.8 molar ratio) and incubated for 4 h at 4°C. This solution was then buffer exchanged to 20 mM sodium acetate with 0.05% Tween at pH 6.0 for cation exchange purification on a 5 ml SP-HP column (GE Healthcare) with a linear gradient from 20% buffer containing 1 M NaCl to 100% of the same buffer at 2 ml/min for 1 h. Fractions containing protein of the expected size of PEG-DI were identified by peaks on a chromatogram at 280 nm and then pooled. The hexahistidine tag was cleaved using FXa as in McDonnell et al (23). This was followed by a single isocratic wash in SEC(16/600, Superdex 75) buffer. For this experiment two different versions of PEG-DI carrying 20kDa PEG and 40kDa PEG were prepared and their properties compared with non-PEG-DI. All preparations were incubated in an endotoxin removal column (Pierce High-Capacity Endotoxin Removal Resin, ThermoScientific) until found to be endotoxin free by the fluorescent endotoxin assay (Hyglos).

Both DI and PEG-DI have been shown to be biologically active in a range of assays, indicating that the expressed DI is correctly folded (4, 21).

Preparation of Proteins β2GPI and OA for Immunization Protocol

β2GPI was isolated from pooled normal human serum, as described in detail elsewhere (24). In brief, human β2GPI was purified using perchloric acid precipitation and affinity chromatography on a heparin-sepharose column (HiTrap HP, GE Healthcare). The eluted material from this first step was then subjected to ion exchange chromatography on a Resource-S column (GE Healthcare). The purity of all β2GPI preparations was confirmed by SDS-PAGE (Mini-Protean TGX 4-20% gel, BioRad) and antigenicity determined by coating ionization-treated polystyrene plates and measuring binding to known anti-β2GPI patient sera in an ELISA procedure as described elsewhere (24). Purified ovalbumin (Sigma-Aldrich) was purchased. All preparations were treated until determined to be free of endotoxin contamination (< 1.0 EU/mL).

Chronic Mouse Model of APS

The method was as described in previous papers (22). Male CD-1 mice (n=5 per group) (Charles River Laboratories, Wilmington, MA) between 3-4 weeks in age (10-15g) were immunized intraperitoneally (IP) with 3 consecutive weekly doses of 0.5 μg of β2GPI in sterile PBS with an equal volume of complete Freund's adjuvant (CFA) at week 0 or incomplete Freund's adjuvant (IFA) at weeks 1 and 2. Negative control mice were injected IP with 0.5 μg of ovalbumin (OA) in CFA or IFA over the 3-dose immunization regimen. The dose of 0.5 μg of β2GPI used in these experiments was based on our previous experiments to optimize the model. We have found that this dose leads to development of anti-β2GPI antibodies in the mice at levels giving optical density of 1.5 to 2.0 in ELISA, similar to results obtained from patients with APS.

Blood was collected retro-orbitally once weekly prior to treatment given at each time point to test development of antiphospholipid antibodies using in-house ELISA assays according to a standardized protocol as previously outlined (24). Terminal surgery to evaluate outcome measures was performed four weeks after initial immunization dose. At 72 hours prior to terminal surgery, a therapeutic dose (20*μg)* of DI peptide construct in sterile PBS or sterile PBS only as a control was given and a second similar dose of the same construct given at 24 hours prior to terminal surgery.

At week 4, mice were anaesthetised and one of the femoral veins was exposed for observation with an approximate 0.5mm segment trans-illuminated using a microscope equipped with a closed-circuit video system. The isolated vein segment was pinched to introduce a standardized injury and thrombus formation and dissolution was visualized and recorded. The treatment groups were as follows: ***1)*** β2GPI *Immunization/PBS treatment (positive control)*, ***2)****OA Immunization/PBS treatment (negative control)*, ***3)****β2GPI Immunization/20 μg non-PEG-DI*, ***4)*** β2GPI *Immunization/20 μg 20 kDa PEG-DI*, ***5)****β2GPI Immunization/20 μg 40 kDa PEG-DI.*

Three outcome measures were assessed, as fully described in previous papers with modifications as outlined below (15). These were:

a) Induced thrombus size: A total of three thrombi were generated in each mouse and the largest cross-sectional area of each thrombus during the formation-dissolution cycle was measured five times and a mean value calculated (in μm^2). Each thrombus dissolves before the next is formed.

b) Tissue Factor (TF) expression in mouse aorta homogenates (measured as described in (b) above). At the end of surgery, mice were perfused with heparin and protease inhibitor cocktail solution and the descending aorta collected (from aortic arch to renal branches). Excess peri-aortic fat was removed and the aorta flash-frozen in heparin and protease inhibitor cocktail and stored at -70°C until processed for TF testing as previously described.

c) Activity of TF in peritoneal macrophages by a solid-phase immunoassay specifically designed to measure mouse TF (Picokine ELISA, Boster Biological). Results were standardized with reference to the protein concentration of lysates (BCA Protein assay, ThermoScientific Pierce) and expressed in $pg/\mu gml^{-1}$ units.

All animals were housed in the viral antibody-free barrier facility at the University of Texas Medical Branch (UTMB). Animal use and care were in accordance with the UTMB Institutional Animal Care and Use Committee (IACUC) guidelines.

Statistical Analysis

Results were expressed as means plus or minus standard deviation as appropriate. A one-way analysis of variance by

ANOVA followed by the Tukey-multicomparison test was used to compare differences among mouse groups.

RESULTS

Immunization of Mice With β2GPI Induces aPL Production

Compared to controls treated with OA, β2GPI-immunized CD1 mice produced significantly greater mean anti-β2GPI levels by week 2 (1.14 ± 0.68 to 1.69 ± 0.39 vs-0.13 ± 0.01OD, p<0.0001) and continued to be significantly elevated over the remaining length of the 4-week test period (p<0.0001). Close to maximum anti-β2GPI levels were attained in week 3 (1.88 ± 0.43 to 2.00 ± 0.38 vs-0.12 ± 0.01OD, p<0.0001) and maintained in week 4 at the time of thrombosis surgery (1.95 ± 0.40 to 2.23 ± 0.24 vs-0.10 ± 0.01OD, p<0.0001). Mean levels of anti-β2GPI at the time of surgery were statistically similar in all groups of mice immunized with β2GPI (p=0.624) (**Figure 1A**).

The associated increase in aCL levels occurred in a similar fashion albeit less robustly. Mean aCL levels significantly increased by week 2 (0.46 ± 0.34 to 0.93 ± 0.61 vs-0.07 ± 0.01OD, p<0.0001) and continued to be significantly elevated over the remaining length of the 4-week test period (p<0.0001). Close to maximum aCL levels were attained in week 3 (0.83 ± 0.22 to 1.14 ± 0.38 vs 0.07 ± 0.01OD, p<0.0001) but waned slightly in week 4 at the time of thrombosis surgery (0.72 ± 0.27 to 0.93 ± 0.28 vs 0.07 ± 0.01OD, p<0.0001). Mean levels of aCL at the time of surgery were statistically similar in all groups of mice immunized with B2GPI (p=0.661) (**Figure 1B**).

PEGylated DI Retains the Ability to Inhibit the Effect of Induced APS-IgG on Development of Thrombosis in a Chronic Mouse Model of APS

In each panel of **Figure 2**, the two columns on the far left show the difference in outcome obtained after a 4-week immunization course with β2GPI (pos control) and OA (neg control) in the absence of any inhibitor. In **Figure 2A**, thrombus size was much larger in positive controls compared to negative controls (1958.3μm^2 vs 645.7μm^2, p<0.0001). Non–PEG-DI treatment significantly reduces thrombus size compared to positive control mice (815.3μm^2, p<0.0001) to a size very similar to that obtained in negative controls (p=0.570). The following two columns show that 20kDa and 40kDa PEG-DI also inhibit thrombosis (818.9μm^2, p<0.0001 and 728.9μm^2, p<0.0001 respectively), with no noted loss of inhibitory activity compared to the non-pegylated construct (p=0.937)

Figure 2B shows TF expression in aorta homogenates of mice immunized with β2GPI and OA. A similar outcome profile was seen with significantly higher TF levels in β2GPI immunized mice, approximately 2.5 times that recorded in OA treated mice (19.3 ± 5.6 vs. 8.1 ± 1.4 pg/μgml^{-1}, p=0.0003). Non-PEG-DI treatment abrogated TF expression in β2GPI immunized mice (p=0.001) to levels similar to control mice. Both the 20KDa and 40kDa PEG-DI also inhibited TF expression with no noted loss of inhibitory activity compared to the non-pegylated construct (p=0.951).

Figure 2C shows TF expression in peritoneal macrophages of immunized mice with a similar outcome profile. Higher TF levels were recorded in β2GPI immunized mice compared to OA treated mice and non-PEG-DI, 20kDa PEG-DI and 40kDa PEG-DI treatment decreased TF expression to levels seen in negative control mice. However, these changes only approached statistical significance (p=0.070).

There was no toxicity noted in any mice treated with the PEGylated or non-PEGylated construct treatments in this chronic active immunization mouse model. In previous experiments using a passive immunization model in which external immunoglobulins were administered, there were noted adverse reactions. These were completely absent in this disease model in which mouse autoantibodies were induced to simulate active APS disease.

DISCUSSION

In this paper, we have built on our previous paper describing the efficacy of PEG-DI in the passive immunization model (21). In that paper, we tested both wild-type (WT) DI and an altered variant containing two point mutations at positions 8 and 9 that we thought might have better inhibitory properties than the wild-type form based on previous experiments on non-PEGylated DI (15). In the event, however, the PEGylated version of this DI variant was not more effective and also had a significant toxicity problem with 40% of mice treated dying. Therefore, in the current experiment, only WT-DI was tested. The other important difference between this paper and the previous one (21) was our use of the chronic mouse APS model (22). Patients with APS have chronically elevated levels of aPL, with higher levels of aPL and triple-positivity for aCL, anti- β2GPI and lupus anticoagulant both being associated with higher risk of thrombosis (25). The presence of these antibodies, however, does not in itself lead to thrombosis. Some patients may be triple-positive for many years without developing thrombosis. This leads to the concept that a "second hit" is required by which some other stimulus such as pregnancy or surgery leads to a new thrombosis in a person predisposed to that by the presence of serum aPL. This chronic mouse model recapitulates what is seen in patients. As shown by our results, immunization with β2GPI leads to persistently elevated levels of aCL and anti- β2GPI in the mice but they do not develop thrombosis till subjected to the femoral vein trauma. It is therefore very encouraging that PEG-DI was just as effective as non-PEG-DI in inhibiting thrombosis in this model and caused no toxicity. As opposed to the passive transfer model that we used previously, the chronic model has a number of advantages including the potential to recapitulate processes such as B and T cell clonal expansion, epitope spreading and break in tolerance. For example, we previously used this model to demonstrate both T cell-dependent and independent aPL production (24).

Although there have been previous attempts to develop therapies for APS based on blocking intermolecular interactions of β2GPI with either antibodies or phospholipids, they have not focused on DI. Whereas DI is the main site of binding for pathogenic anti-β2GPI

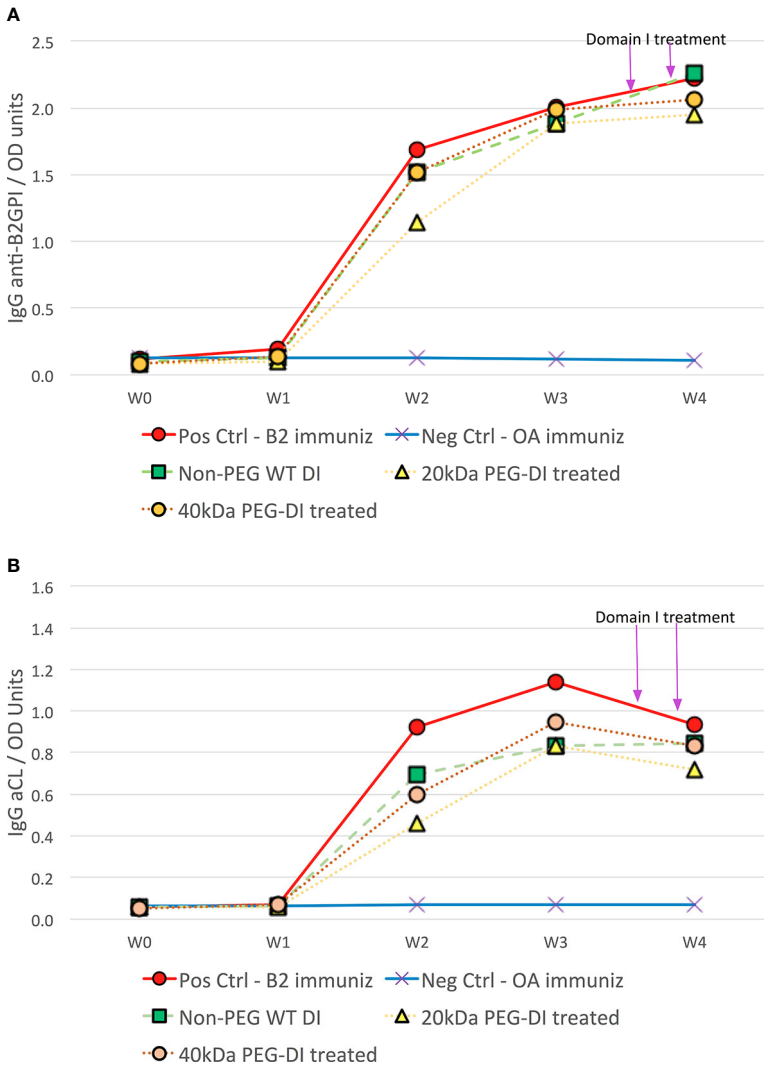

FIGURE 1 | Antiphospholipid antibody development in chronic mouse model of APS disease. CD1 mice immunized with β_2GPI produced significant titers of anti-β_2GPI **(A)** and aCL **(B)** over the 4-week period compared to corresponding OA immunized negative controls. At the time of thrombus surgery at week 4, aCL and anti-β_2GPI titers in positive control β_2GPI-immunized mice were similar to those in β_2GPI-immunized mice treated with non-PEG-DI, 20kDa PEG-DI and 40kDa PEG-DI prior to surgery.

antibodies, the C-terminal DV interacts with anionic phospholipids on cell surfaces *via* a lysine-rich patch on the domain (26). It is believed that this interaction of surface phospholipids with the antibody- β2GPI complex leads to cellular effects critical to the pathogenesis of APS (6). Therefore, several groups have attempted to block the phospholipid-DV interaction rather than the antibody-DI interaction in developing new therapies for APS.

TIFI is a 20 amino acid peptide that has homology to DV and inhibits the binding of DV to phospholipids. Vega-Ostertag and colleagues demonstrated that TIFI blocked binding of β2GPI to human umbilical vein endothelial cells *in-vitro* and also inhibited thrombosis caused by passive administration of IgG from patients with APS in the same model that we previously used (27). A control peptide VITT (containing similar amino acids to TIFI but in a different order) did not inhibit thrombosis. A different group, in Italy, studied the efficacy of TIFI in a mouse model of obstetric APS. They showed that TIFI inhibited binding of APS-IgG to human trophoblast cells whereas VITT did not (28) Furthermore, when pregnant C57Bl6 mice were treated intravenously with either monoclonal human aPL or control IgG from healthy people, only the aPL-treated mice suffered fetal loss and reduced fetal and placental weight. All these pathogenic effects of aPL were reduced by treatment with TIFI at days 0,5 and 10 before sacrifice at Day 15 whereas they were not reduced by VITT (28). TIFI, however, is a small molecule that would suffer the same problem of short half-life that we have described for native DI. No-one has yet published any data on forms of TIFI that have been modified to increase serum half-life.

An alternative approach has been described in a series of papers from the group of Kolyada and colleagues (29–32). They have taken advantage of the fact that the A1 domain of the apolipoprotein E

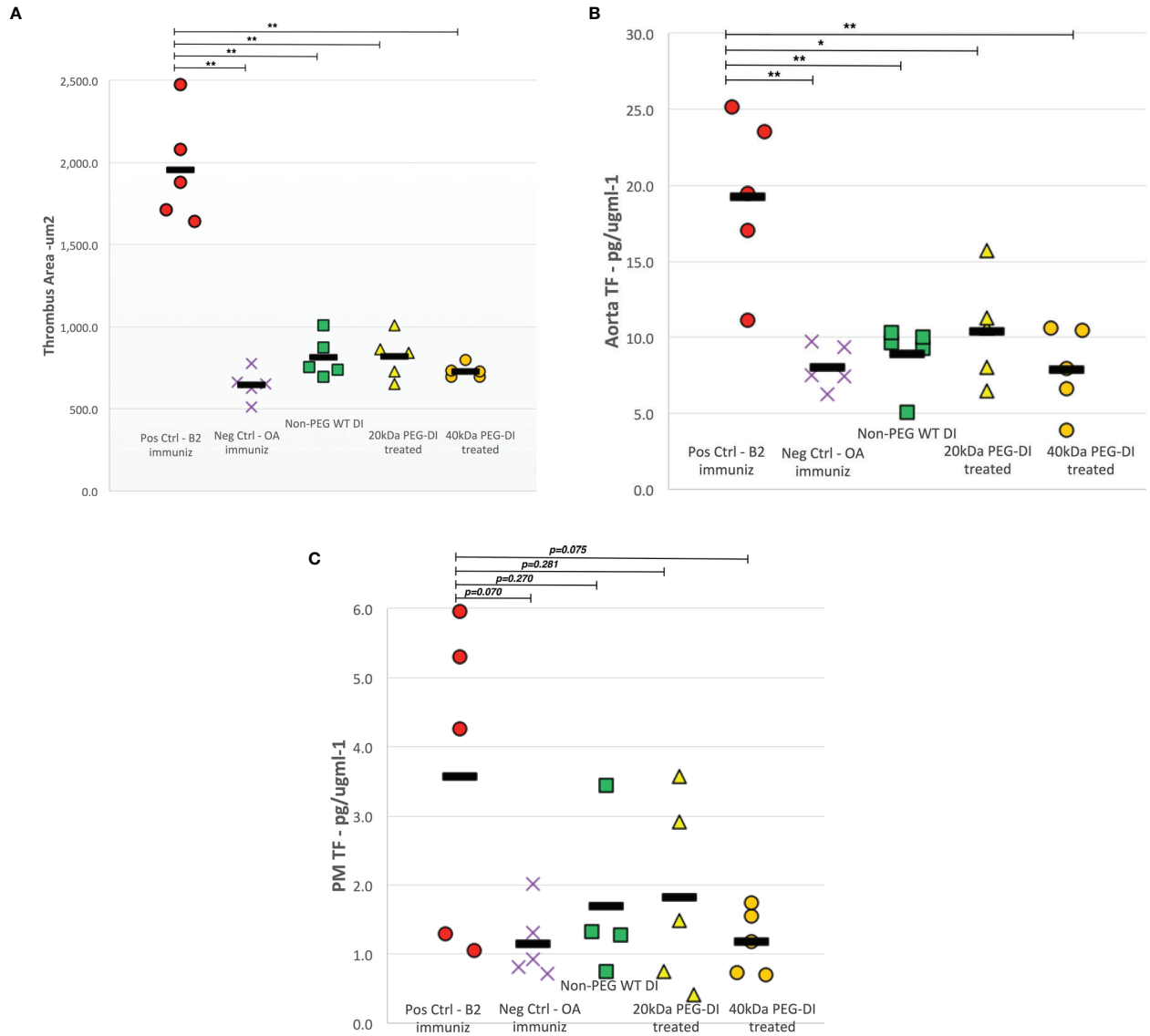

FIGURE 2 | Thrombus area, aortic and peritoneal macrophage tissue factor levels in chronic APS mouse model. Both thrombus area **(A)** and aortic TF **(B)** levels decreased significantly in β_2GPI-immunized mice treated with non-PEG-DI, 20kDa PEG-DI and 40kDa PEG-DI compared to β_2GPI-immunized positive control mice. The abrogated levels of thrombus development and aortic TF were similar to that seen in negative control mice. While there was a trend of decreased peritoneal macrophage TF **(C)** levels in all domain I treated mice, the noted reductions were not statistically significant. (*p<0.01, **p<0.001).

receptor 2 (ApoER2) binds to DV. By creating a dimer of A1 in which two A1 molecules are joined by a flexible linker, they created an agent that blocked binding of β2GPI-anti β2GPI complexes but not of non-complexed β2GPI to CL (29). They then showed that the same agent reduced thrombosis induced by a laser in both an autoimmune (NZWxBXSB1)F1 mouse model of APS and in healthy strain mice that had been infused passively with APS-IgG (30). They also showed that a point mutant of A1-A1 dimer had better inhibitory properties *in-vitro* than wild-type A1-A1 (31). The A1-A1 dimer, however, has the same problem of short serum half-life that pertains to DI and TIFI. In the mouse model experiments, 84% of A1-A1 had been lost from the blood within an hour (30). In a subsequent experiment in (NZWxBXSB1)F1 mice, this problem was counteracted by use of a subcutaneous osmotic pump to deliver the agent continuously over 2-4 weeks (32). A reduction in the blood pressure of the mice was reported, but thrombosis was not an outcome of this study (32). It is unlikely that patients would accept use of such a device for a treatment that is essentially preventative, and there are no reports of chemical modification of A1-A1 to increase serum half-life.

In conclusion, there is a pressing need for new therapies for APS. The current standard of care with vitamin K antagonist anticoagulants such as warfarin is fraught with difficulties such as risk of haemorrhage. The initial promise of direct oral

anticoagulants has unfortunately not been fulfilled. Therapies that target the interaction of DV of β2GPI with phospholipids have shown encouraging results in mice, but none have been modified to give serum half-life that would allow therapeutic use in patients. Our work in developing a PEGylated inhibitor of the anti- β2GPI-DI interaction is therefore important and we have now shown its efficacy in both a chronic autoimmune and an acute passive transfer model of APS. The mechanism of the effect is not yet clear and it remains to be demonstrated whether PEG-DI alters production or serum levels of anti-DI antibodies in these mice.

It is important to recognize that up to 20% of anti- β2GPI antibodies in patients with APS bind epitopes outside DI and so PEG-DI will not be effective in all patients. We have not yet shown an improvement in serum half-life of the PEGylated variant, though in the previous experiment (21) the inhibitory effects of the agent were demonstrable 48 hours after the last dose of PEGylated DI. Formal pharmacokinetic and toxicology experiments will now be important.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Texas Medical Branch Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

TM carried out laboratory work, developed methodology and produced PEG-DI. RW carried out mouse work in Texas and co-wrote the paper. CP developed methodology and gave intellectual guidance. IG gave intellectual guidance. EG and AS gave direction and support regarding experiments in Texas. ZR-P carried out practical work in Texas regarding mouse models. AR gave intellectual input, co-wrote the paper and supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: TM, CP, IG, and AR are all named co-inventors on a patent filed in the US for PEG-DI.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Malignant Isolated Cortical Vein Thrombosis as the Initial Manifestation of Primary Antiphospholipid Syndrome: Lessons on Diagnosis and Management From a Case Report

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Background: Antiphospholipid syndrome (APS) with isolated cortical vein thrombosis (ICoVT) is an extremely rare but potentially malignant entity. It is particularly challenging to diagnose APS-related ICoVT because of the non-specific clinical manifestations and the frequent absence of typical neuroimaging. Moreover, there is currently limited knowledge on the clinical features and management strategies for the condition. Delays in diagnosis and treatment may lead to life-threatening consequences.

Case Presentation: We present a rare case of a 74-year-old Chinese woman who presented with sudden onset of headache and right arm weakness that mimicked acute ischemic stroke. Her initial computed tomography was unremarkable, and intravenous thrombolysis was performed. Serial neuroimages confirmed ICoVT 4 days after symptom onset, and low-molecular-weight heparin (LMWH) was started at a dose of 0.4 ml twice per day, according to the 2019 Chinese guidelines. The workup for the predisposing causes of ICoVT revealed triple positivity APS. LMWH dose was adjusted according to the anti-Xa chromogenic assay. However, the patient's condition deteriorated rapidly, and there was a progressive enlargement of the venous infarction despite treatment with anticoagulants. Transtentorial herniation developed on day 12, and decompressive craniectomy was immediately performed. The patient's symptoms did not improve significantly after surgery, and she remained aphasic and hemiplegic at the 3-month follow-up, with a modified Rankin Scale score of 5.

Conclusion: ICoVT is a rare yet potentially fatal manifestation of APS, and its diagnosis and treatment are extremely challenging. Timely diagnosis, prompt treatment, and close monitoring are essential to improve the clinical prognosis of patients with APS-related ICoVT.

Keywords: cortical vein thrombosis, antiphospholipid syndrome, anticoagulation, decompressive craniectomy, magnetic resonance, case report

INTRODUCTION

Isolated cortical vein thrombosis (ICoVT) is a relatively rare and poorly understood phenomenon that represents <0.1% of all stroke entities and 6%–8.7% of all cerebral venous sinus thrombosis (CVST) cases (1, 2). ICoVT is defined as thrombosis that only involves the cortical veins (CoVs) without occlusion of the dural venous sinuses and deep veins (1). The diagnostic challenge for ICoVT is attributed to the non-specific clinical manifestations, frequent anatomical variations in CoVs, and difficulties in determining ICoVT using conventional imaging modalities, such as magnetic resonance imaging (MRI) and magnetic resonance venography (MRV). In contrast to patients with CVST, patients with ICoVT often have parenchymal involvement (81% vs. 40.1%, respectively) and hemorrhagic lesions (46% vs. 13.4%, respectively) (3, 4). Although most ICoVT patients achieve a favorable prognosis, delays in diagnosis and treatment can lead to life-threatening consequences (2, 3). Additionally, it is important to note that CoV involvement is strongly associated with malignant CVST, which is defined as a supratentorial cortical venous infarction alongside malignant brain edema, leading to transtentorial herniation either at onset or after exacerbation (5).

Antiphospholipid syndrome (APS) is a non-inflammatory autoimmune disease characterized by persistent circulating antiphospholipid (aPL) antibodies (6, 7). Common clinical features of APS include venous thromboembolism, stroke, and recurrent abortion (6). APS contributes to 6%–17% of CVST cases, yet it is rarely considered an etiology of ICoVT (3, 8, 9). To date, only three cases of ICoVT have been reported to be associated with APS (10–12). Strikingly, APS-related CVST patients appear to have higher rates of disability and mortality than those of CVST patients without APS, especially APS-related ICoVT patients, in whom one out of the three reported cases experienced malignant cerebral edema and underwent decompressive hemicraniectomy (10).

Here, we present an extremely rare case of a primary APS patient who presented with malignant ICoVT as an initial presentation, whose condition rapidly deteriorated despite anticoagulation treatment. This case highlights our concern among clinicians for this rare yet potentially fatal disease. We discuss the lessons that can be learned from the diagnosis and management of this rare disease and how such lessons can aid in improving patient care and outcomes. Furthermore, we discuss the importance of radiologic monitoring and anti-Xa activity measurement to guide anticoagulant therapy for patients experiencing clinical deterioration.

CASE PRESENTATION

A 74-year-old Chinese woman (gravida 3 para 3) was admitted to the emergency department with sudden onset of headache and right arm weakness for 2.5 h. She had a history of well-controlled hypertension and hyperlipemia, for which she took 5 mg of amlodipine daily and 10 mg of atorvastatin daily as instructed by her primary care provider. She had no family history of thrombotic disease and no history of venous thrombosis. She also denied any history of risk factors for thrombosis (e.g., oral contraceptives, malignancy, and autoimmune diseases).

On admission, the patient's vital signs were within normal limits except for a slightly elevated blood pressure of 141/89 mmHg. Her weight was 63 kg. Neurological examination showed Medical Research Council (MRC) grade 3 for the right arm. The National Institute of Health stroke scale (NIHSS) score at admission was 2. Non-contrast computed tomography (NCCT) was performed, and the radiologist reported no acute abnormalities (**Figure 1A**). The emergent blood assay revealed no abnormalities except for an elevated D-dimer level at 2.4 μg/ml (normal range, 0–0.5 μg/ml). Contraindications for intravenous thrombolysis (IVT) were excluded. After obtaining informed consent from the patient, IVT with 54 mg alteplase was administered 3 h post-onset. The neurological deficits and headache progressively improved, and the NIHSS score 1 h after IVT was 0.

On day 2, the patient complained of headache and weakness of the right extremity (MRC grade 4/5). Her D-dimer level was 4.87 μg/ml. Other laboratory workups were negative and included blood routine tests, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), liver and renal function, lipids panel, glycated hemoglobin, homocysteine, infection panel, and tumor markers (e.g., carcinoembryonic antigen, alpha-fetoprotein, neuron-specific enolase, and cancer antigen-125). A repeat CT at 24 h showed no hemorrhage (**Figure 1B**). Aspirin (300 mg/day) and rosuvastatin (20 mg/day) were administered as secondary prevention.

The brain MRI on day 3 revealed punctate abnormalities [hyperintensity on T1-weighted imaging (T1WI) and fluid-attenuated inversion recovery imaging (FLAIR)] that neighbored the cerebral falx and left frontal sulcus (**Figures 1E, F**). However, diffusion restrictions, which are the key parameters for identifying cerebral infarctions, were absent in the parenchyma on diffusion-weighted imaging. After consultation with radiologists, a convexity subarachnoid hemorrhage (cSAH) was considered. Given that the patient was clinically stable, aspirin was continued. CT angiography was performed on day 4 and did not find any aneurysm or arteriopathy. Aneurysmal subarachnoid hemorrhage (SAH) was excluded. However, filling defects of CoVs and compensatory expansion of the adjacent veins were present (**Figures 1C, D**). We then re-evaluated the existing radiographic data and found that punctate hyperdensities within the left frontal sulci, which were ignored on the initial NCCT scans, were consistent with the punctate abnormalities on MRI. The punctate hyperdensities were reduced in the repeated CT acquired 24 h after IVT, which suggested thrombi in the cortical veins. Moreover, lower-extremity intermuscular venous thrombosis was identified by ultrasonography. Thus, ICoVT was highly suspected.

The patient complained of severe headache and progression of right limb weakness (MRC grade 3/5). The MRI on the night of day 4 showed a venous infarction in the frontal lobe (**Figure 1G**). Susceptibility-weighted imaging (SWI) showed curvilinear, serpentine-exaggerated susceptibility within the sulcus, which indicated thrombosed veins in the acute phase (**Figure 1H**). The original MRI abnormality was SWI hyperintensive, which

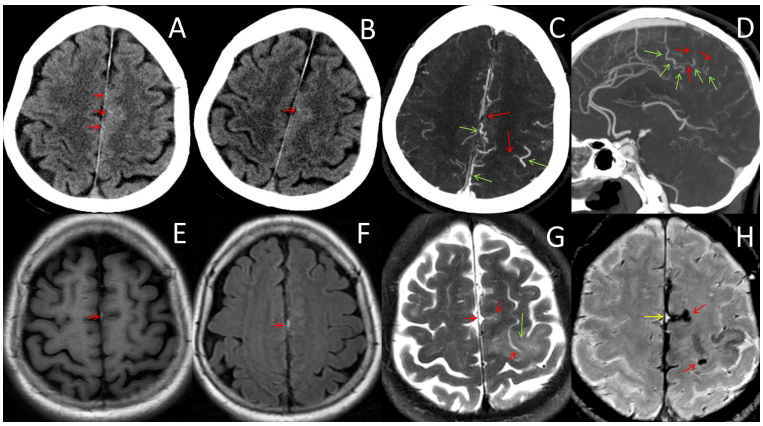

FIGURE 1 | Neuroradiological data from onset to day 4. **(A, B)** CT images before and after intravenous thrombolysis showed no abnormalities except punctate hyperdensities within the left frontal sulci (red arrows). **(C, D)** CT angiography on day 4 showed filling defects of cortical veins (red arrows) and compensatory expansion of the adjacent veins (green arrows). **(E, F)** MRI on day 3 showed hyperintense T1WI and FLAIR lesions neighboring the falx cerebri and the left parietal cortex (red arrows), which were initially diagnosed as subarachnoid hemorrhages, indicating subacute thrombosis in the cortical veins. **(G)** MRI on day 4 showed venous infarction in the frontal lobe (green arrow) and the absence of the flowing-void effect in the cortical veins (red arrows). **(H)** Susceptibility-weighted imaging on day 4 showed curvilinear, serpentine, and exaggerated susceptibility within the sulcus, which was suggestive of thrombosed veins during the acute (red arrows) and subacute phases (greed arrow).

corresponded to subacute vein thrombosis. Therefore, ICoVT was confirmed.

The workup for acquired and inherited hypercoagulability [e.g., antinuclear antibody, antineutrophil cytoplasmic antibodies, rheumatoid factor, procalcitonin, protein C, protein S, antithrombin III, coagulation factor activities panel, thrombophilia gene panel, lupus anticoagulant (LA), anticardiolipin (aCL)], and anti-β2-glycoprotein-I (aβ2GPI) antibodies] was performed on the morning of day 5 before the patient underwent anticoagulation. Additionally, screening for an underlying malignancy was initiated, including chest CT, transthoracic echocardiography (TTE), abdominal ultrasound, and transvaginal ultrasound. However, no evidence of malignancy was found. In the absence of infective symptoms, systemic infection (e.g., infective endocarditis, pulmonary infection, and urinary infection) was excluded according to normal white blood cell count, urine analysis, CRP, ESR, procalcitonin TTE, and chest CT. Laboratory workup reported the next day were positive for LA (dilute Russel viper venom time normalized ratio, 1.78; normal range, 0.7–1.2), aCL IgG (77 IU/ml; normal range, <20 U/ml), aCL IgA (63 IU/ml; normal range, < 20 U/ml), aβ2GPI IgG (89 IU/ml; normal range, <20 U/ml), aβ2GPI IgA (65 IU/ml; normal range, <20 U/ml). Factor VIII, Factor VII, and von Willebrand factors significantly increased by 225% (normal range, 50%–150%), 212% (normal range, 50%–150%), and 160% (normal range, 50%–150%), respectively.

Aspirin was stopped on the morning of day 5, and low-molecular-weight heparin (LMWH) was started at a dose of 0.4 ml every 12 h according to the 2019 Chinese guidelines for clinical management of CVST (13). On the dawn of day 6, the patient experienced an episode of generalized tonic–clonic seizures, which was controlled with diazepam and levetiracetam (0.5 g twice per day). Electroencephalogram showed epileptiform discharges in the left frontal lobe. An enlargement in the venous infarction was observed on the re-examination CT (**Figure 2A**). According to the institutional periprocedural protocol, lumbar puncture was scheduled 24 h after the final administration of LMWH. The cerebrospinal fluid pressure was 160 mmH_2O, and cerebrospinal fluid analyses were all within the normal range. Thus, intracranial infection, SAH, and intracranial hypertension were subsequently excluded. LMWH was restarted 4 h after the lumbar puncture.

MR black-blood-thrombus imaging (MRBTI) on day 7 showed vein thrombosis that extended proximally to the superior sagittal sinus (**Figure 2E**). MR venography showed filling defects of the left frontal and partial cortical veins (**Figure 2F**). The patient's condition deteriorated rapidly, and she developed drowsiness and lethargy; moreover, the muscle strength of her right limbs dropped to grade 1. We employed a multidisciplinary approach combining neurointervention, rheumatology, and hematology consultations. The diagnosis of ICoVT and possible APS was made, and hydroxychloroquine was additionally prescribed. LMWH was recommended to be adjusted with an anti-Xa chromogenic assay. Digital subtraction angiography (DSA) was scheduled to evaluate whether direct intrasinus thrombolysis should be performed.

On day 8, peak anti-Xa was 0.4 IU/ml, and LMWH was increased to 0.6 ml twice per day. DSA demonstrated multiple thromboses of superior and internal cerebral veins without sinus thrombosis (**Figures 2G, H**). However, thrombolysis was negated by the neurointerventionalist. The conditions continued to worsen progressively, with enlargement of the venous infarction in the CT on day 10 (**Figure 2B**). Peak anti-Xa was 0.71 IU/ml, and a high-intensity LMWH dose of 0.8 ml twice per day was taken with a goal of achieving 0.8–1.0 IU/ml.

On day 12, the patient progressed to a coma, and the MRI showed a large intracranial hemorrhage (ICH) in the left frontoparietal lobe and transtentorial herniation (**Figure 2C**).

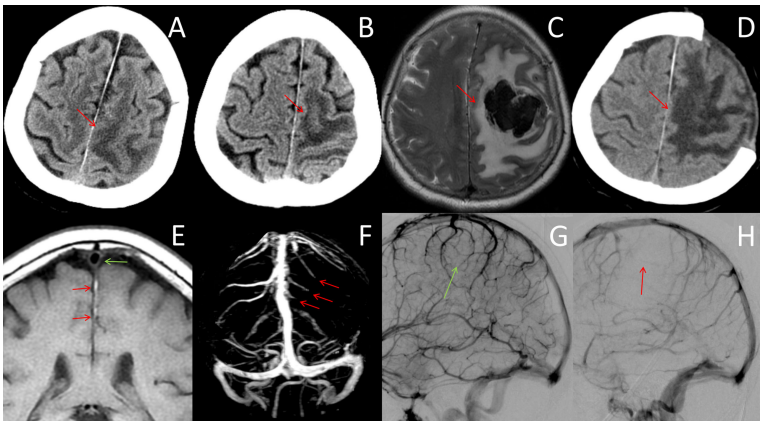

FIGURE 2 | Neuroradiological data after day 4. **(A, B)** CT images on days 6 and 10 showed progressive enlargement of the venous infarction lesion in the frontoparietal cortex (red arrows). **(C)** MRI on day 12 showed venous infarction lesions with a large acute hematoma in the left frontoparietal lobe, which resulted in transtentorial herniation (red arrows). **(D)** CT scan after decompression craniectomy showed large areas of low-density lesions (red arrow). **(E)** MR black-blood-thrombus imaging (MRBTI) on day 7 showed vein thrombosis that extended proximally to the superior sagittal sinus (red arrow). The superior sagittal sinus was free of thrombosis (green arrow). **(F)** MR venography showed filling defects of the left frontal and partial cortical veins (red arrows). **(G, H)** DSA on day 8 showed normal venous flow of the right hemisphere (**G**, green arrow) compared with multiple thromboses in the left superior and internal cerebral veins of the left hemisphere (**H**, red arrows).

Decompressive craniectomy was performed immediately (**Figure 2D**). LMWH was restarted 3 days after the operation at a dose of 0.4 ml twice per day based on the risk of hemorrhage. Warfarin was given 3 weeks after craniectomy. The patient's symptoms did not improve significantly after surgery and was thus transferred to a rehabilitation hospital. At the 3-month follow-up, serum aCL IgG (51 IU/ml) and aβ2GPI IgG (71 IU/ml) were positive, and a diagnosis of primary APS was established. LA was not retested because of long-term warfarin therapy. The patient remained aphasic and hemiplegic. Her modified Rankin Scale (mRS) score was 5. The timeline of the case is showed in the **Figure 3**.

DISCUSSION

It is well-established that ICoVT is the rarest subtype of CVST that presents as an isolated occlusion of anastomotic surface veins (2, 14). It remains a poorly understood condition without established guidelines for rapid identification or precise treatment. Its propensity to mimic not only symptoms but also neuroradiological manifestations of numerous other diseases makes diagnosis particularly challenging (14). Misdiagnoses include acute ischemic stroke (AIS), cSAH, glioma, cerebral parasitic disease, and even multifocal leukoencephalopathy (15). Symptoms and signs such as seizures and motor and sensory disturbances are common in ICoVT, especially in the early stages, which results in the misdiagnosis of the underlying ICoVT (16). As observed in our patient, the sudden onset of right arm weakness without the presence of ICH was consistent with a diagnosis of AIS. Owing to the narrow time window of IVT, differential diagnosis can be challenging. The European Stroke Organization (ESO) guidelines for CVST recommend that D-dimer should be measured before neuroimaging in patients with suspected CVST. Meng and colleagues enrolled 233 patients with an unexplained headache who visited the emergency room. The average D-dimer level was significantly higher in CVT patients (987.7 ± 324 mg/L) than in patients in the mimic group (343.23 ± 102 mg/L), resulting in high sensitivity (94.1%) and specificity (97.5%). Although ICoVT is limited to focal segments of surface veins, D-dimer has also been shown to be sensitive (16). The marked increase in D-dimer level in our case may provide a clue for the diagnosis of ICoVT. The suspicion of venous thrombosis should be raised because of the early onset of headache and the marked increase in D-dimer level, given that these features are rarely seen in patients with lacunar stroke (17). The accuracy of D-dimer for diagnosing acute lacunar stroke is well established, with reported rates of 88% with D-dimer alone and 98% with the conjunctive use of D-dimer and the Oxfordshire Community Stroke Project classification (18). However, elevation of D-dimer is observed in most thrombotic vascular diseases. Moreover, it should be noted that D-dimer levels may rise in certain pathological states besides thrombosis, such as cancer, infection, and liver disease. D-Dimer may also be influenced by various factors, such as age, race, sex, and medications (19). Therefore, D-dimer should be used as part of a comprehensive diagnostic tool for CVST but not as a stand-alone test. Because of the low cost, convenience, and speed of D-dimer measurement, we still recommend to measure D-dimer when suspecting CVST. A high level of clinical suspicion is required to consider ICoVT in patients who present with increased D-dimer level and symptoms of focal neurological deficit and seizures, particularly in young patients and pregnant/puerperal women.

It is not surprising that ICoVT shares similar sex and age distribution, predisposing factors, and clinical presentations as CVST (2, 14). Although female sex predilection and young

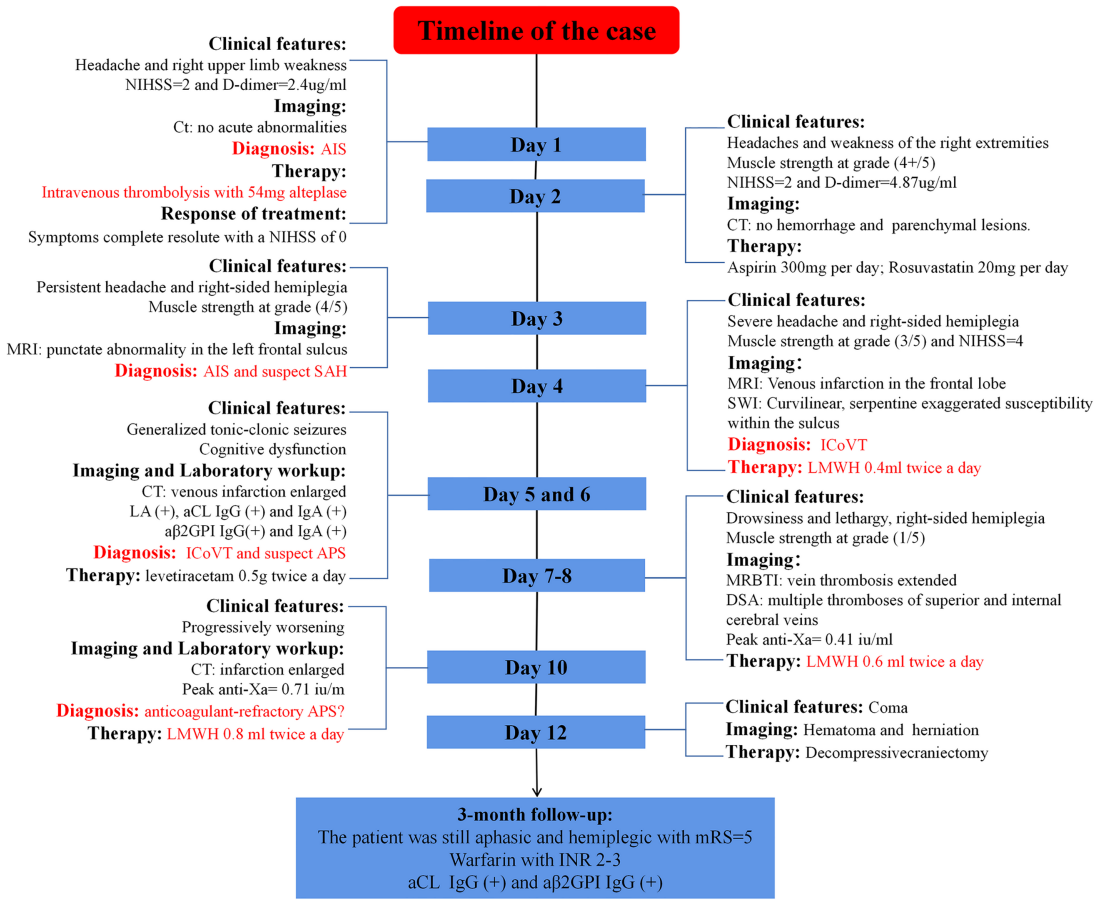

FIGURE 3 | Timeline of the present case.

individuals are well-established factors (2, 14), there are some differences in predisposing factors between ICoVT and CVST. Murumkar and colleagues recently conducted the largest retrospective observational study to date of ICoVT involving 28 patients. They concluded that iron deficiency anemia, hyperhomocysteinemia, infection, and female hormonal imbalance were the top 4 risk factors (2). Systemic autoimmune diseases were rarely considered the cause of ICoVT in their cohort, and only one patient with mixed connective tissue disease was reported. This phenomenon is in line with a previous case series report and a systematic review (3, 20). Song and colleagues reported a similar phenomenon in patients with acute cortical vein thrombosis. Only four (17.4%) patients had underlying autoimmune diseases (16), two of whom had APS but not ICoVT and were identified as having abnormalities in the cerebral sinus using MRBTI. Despite the scarcity of publications, physicians should certainly not neglect APS as a trigger factor for ICoVT given the potential malignant nature of APS-related ICoVT.

APS is an immune-mediated thrombophilic disorder propelled by a heterogeneous group of persistent circulating aPLs and associated proteins (9, 21). APS is characterized by recurrent vascular thrombosis, pregnancy morbidity, and non-criteria manifestations, such as livedo reticularis, thrombocytopenia, and valve disease (6, 9). Well-established antiphospholipid antibodies include aCL, aβ2GPI, and LA. The diagnosis of APS still relies on the 2006 Sydney criteria (6). As the clinical events of thrombosis can occur in the vascular beds of any size and organ, the clinical presentation of APS varies enormously (9). APS contributes to 6%–17% of CVST patients across cohort studies (9). However, CVST is a relatively rare presentation of APS, with a reported prevalence of only 0.7% (9, 22). A recent systematic review on APS-related CVST patients showed that CVST predominantly affects young women and involves the superior sagittal sinus and lateral venous system (60% and 69%, respectively) (23). Extensive thrombosis is a vital characteristic of APS-related CVST patients, with an incidence of 60% (23). Only 1.8% of APS-related CVST are identified as ICoVT (23). Thus, it is not difficult to understand that ICoVT is an extremely rare presentation of APS that depends on the propensity of APS to develop into widespread vascular thrombosis. To the best of our knowledge, ICoVT has occurred in only four patients with APS to date, including our case (10–12). The detailed information of the cases is listed in the **Table 1**. It is vital to highlight that half of these patients presented with malignant ICoVT despite undergoing anticoagulation.

TABLE 1 | Clinical and neuroradiological characteristics of APS-related ICoVT.

	Present case	Polster et al. (10)	Numata et al. (11)	Miranda et al. (12)
Age and sex	73/Female	16/Female	45/Male	29/Female
Risk factors	Hypertension, hyperlipemia	Post-partum	Tadalafil ingestion	OC, smoking
Clinical course	Acute	Acute	Acute	Acute
Initial symptoms	Headache, focal neurological deficit	Focal neurological deficit	Posterior headache	Focal neurological deficit focal epilepsy and GTCS
Initial diagnosis	Acute ischemic stroke	Acute stroke	Tension headache or cerebrospinal fluid hypovolemia	Not mentioned
Sequential symptoms	GTCS, consciousness disturbance	GTCS, consciousness disturbance	GTCS, consciousness disturbance	GTCS
Involved veins	Left superior and internal cerebral veins	Left superior cerebral veins	Right parietal CoV	Left frontal CoV
Parenchymal changes	VI in frontoparietal cortex, then converted to hemorrhagic VI	Hemorrhagic VI in frontoparietal cortex	VI in right parietal cortex	VI in left prefrontal cortex
Neuroradiological signs of ICoVT	CT: punctate hyperdensities in the sulci MRI: subacute CoV thrombus SWI: exaggerated susceptibility within the sulcus	CT: cord sign CTV: filling defect of CoV	CT: CT cord sign SWI: SWI cord sign	GRE T2WI: cord sign
DSA	Filling defect of CoVs	Not performed	Not performed	Not performed
APS Criteria	Sydney	Sapporo	Sydney	Not mentioned
APS Antibodies	LA, aCL, IgG, and IgA, aβ2GPI IgG and IgA	LA	aCL, IgG, and aβ2GPI IgG	aCL IgM
Laboratory abnormalities	Elevated D-dimer	No	Elevated D-dimer	No
Anticoagulation	Low-molecular-weight heparin, then warfarin	Heparin, then warfarin	Heparin, then warfarin	Heparin, then warfarin
Complications	Hemorrhage, brain herniation	DIC, brain herniation	No	No
Decompression craniectomy	Yes	Yes	Not performed	Not performed
Recurrence	No	No	No	No
Prognosis	Poor, mRS score of 5 at 3 months	Poor, mRS score of 4 at 6 months	Excellent	Excellent

aβ2GPI, anti-β2-glycoprotein-I antibody; aCL, anticardiolipin antibody; CoV, cortical vein; CT, computed tomography; CTV, computed tomography venography; GRE, gradient-echo images; GTCS, generalized tonic–clonic seizures; LA, lupus anticoagulant; MRI, magnetic resonance imaging; mRS, modified Rankin Scale; OC, oral contraceptives; VI, venous infarction.

In addition, the mortality of APS-related CVST is likely to be higher (6.4%–15.8%) than those without APS (<5% in most studies) (23–25). This suggests that patients with APS are more likely to have a poor outcome and require close monitoring, especially high-risk patients with high adjusted global APS (aGAPSS) scores (26).

Because of the non-specific clinical manifestations of ICoVT, neuroimaging is the cornerstone of ICoVT diagnosis (2, 27). However, precise imaging diagnosis is challenging owing to the nature of CoVs, which include their relatively small size, anatomical variations, and collateral circulation. There are many lessons to be learned from our case in regard to neuroimaging diagnosis. First, the punctate hyperdensities within the left frontal sulci were ignored on the initial NCCT scans. NCCT is usually the first diagnostic tool used, especially for patients in the emergency department. The CT "cord sign" is the direct visualization of the thrombosed cortical veins, which present as cord-like hyperdensities in the cerebral sulcus (15, 28). This sign is rare and has low diagnostic sensitivity for ICoVT. Indeed, in a cohort of 71 patients, no patient presented this sign (28). We speculate that punctate hyperdensities in the sulci are equivalent to the cord sign as a result of the limited amount of thrombosis in cortical veins. Other indirect signs of NCCT, such as narrowing of the neighboring sulci and sulcal hemorrhage, are also uncommon (28). Therefore, ICoVT is easily overlooked on NCCT, and patients with predisposing risk factors or an increased D-dimer level require further workup despite normal NCCT. Interestingly, we observed an improvement in the punctate hyperdensities on NCCT after IVT, which suggested partial dissolution of the thrombus. Second, ICoVT mimics cSAH on MRI, which led to a delay in diagnosis and treatment in our case. The cSAH has been reported as a complication of IVT; thus, it must be considered a differential diagnosis of post-IVT headache. The repeat CT showed improvement in punctate hyperdensities, which made the diagnosis of cSAH less likely. Subacute ICoVT (5–14 days) is difficult to differentiate from cSAH because of the similar radiographic appearance when parenchymal involvement is absent (29). Subacute ICoVT and cSAH are usually observed along the sulci and are shown as hyperdensities on NCCT and hyperintensities on FLAIR images. On conventional MRI, acute ICoVT is isointense on T1WI, hypointense on T2WI and SWI, whereas subacute ICoVT is hyperintense on T1WI and T2WI and is also called the hyperintensive vein sign. SWI can help with the differential diagnosis of SAH. Third, vascular lesions should be carefully evaluated, rather than focusing on parenchymal lesions. Changes in parenchymal lesions always lag behind the development of the underlying venous thrombosis. Although the venous infarction was localized to the left parietal cortex, DSA revealed multiple thromboses of CoVs in our case, which resulted in malignant complications. These imaging findings should be considered as warning signs of clinical deterioration. Given that supratentorial cortical thrombosis is more vulnerable to transtentorial herniation, more aggressive therapy may be required to prevent disease progression (30). Careful imaging analysis is particularly critical for clinical decision-making.

The diagnosis of ICoVT remains relatively difficult despite progress in imaging techniques (2). Non-contrast MRI may help detect thrombosis in small peripheral cortical veins based on these signs but only during the acute to subacute phases (27). In our patient, the MRI acquired on day 3 showed hyperintense T1WI, T2WI, and FLAIR lesions neighboring the falx cerebri and the left parietal cortex, which suggested subacute thrombosis in the CoVs. The small filling defect of the peripheral CoVs on contrast CT enabled us to confirm the presence of thrombosis; however, it could have been easily missed by the radiologists. Contrast CT is reported to have low sensitivity for small vein occlusions and is rarely used in clinical practice (2). Post-contrast TIWI demonstrated fair sensitivity to contrast CT in a previous study, with only limited value in the detection of thrombosis in the venous sinus and large CoVs (2). Previously, DSA was the gold diagnosis standard for ICoVT owing to its ability to detect slow blood flow and filling defects in CoVs. However, its diagnostic value in ICoVT is somewhat questionable, with a reported diagnostic rate of approximately 14.3%–47% (16). Contrast-enhanced MRV has been reported to be the best routine diagnostic tool for ICoVT, with a diagnostic rate of 45%–73% (3, 14). Despite the shortcomings in demonstrating distal cortical vein thrombosis and distinguishing anatomic venous variations, contrast-enhanced MRV is a fast, non-invasive, and recommended technique. SWI can detect different forms of iron in the tissue and is effective at visualizing CoV thrombosis and venous stasis surrounding the thrombosis (2, 14). However, its pitfalls near bone interfaces and prior hemorrhage should be excluded carefully (2). Recent advances in MRBTI have enabled the qualitative evaluation of the pathological stages of the venous thrombus and the quantitative assessment of the thrombus load (2, 16). MRBTI has excellent value in identifying cortical vein thrombus, with a sensitivity rate of 100%; moreover, it is highly efficient at distinguishing ICoVT from CoVT comorbid with CVST (16).

LMWH or unfractionated heparin followed by oral antagonists for an individualized duration according to predisposing causes is recommended by the current clinical guidelines (9, 13). However, the therapy strategy, including drug choice, dose intensity, and treatment duration, remains controversial in the context of different comorbid diseases. For example, thrombocytopenia is a common yet crucial complication of APS, which raises a dilemma for the management of APS-related CVST (7, 9). Furthermore, there are several dilemmas for APS-related ICoVT. First, ICH is particularly common in APS-related ICoVT, which raises the question of when to start and how to manage anticoagulation in the presence of ICH. The 2019 Chinese guidelines and the ESO guidelines state that minor ICH is not an absolute contradiction to anticoagulation. The evidence for this recommendation is derived from two randomized control trials that reported mean delays in anticoagulation treatment of 10.9 and 32.5 days after initial symptom onset, although they only included patients with minor ICHs who had been stable for several days (10). Thus, there is currently a lack of evidence on the optimum timing for starting anticoagulation. The early initiation of anticoagulation in patients with hemorrhagic CVST has been called into question, especially those with a large or expanding ICH. Previous studies have reported that the presence of early ICH is strongly associated with expanding or new-onset ICH following intravenous

unfractionated heparin treatment (23.7% vs. 10.9%) (31). Considering the possibility of further aggravation of the ICH, hemorrhagic CVST remains a therapeutic challenge. Second, CoV thrombosis tends to be malignant and life threatening despite anticoagulation. Therefore, understanding how to monitor and manage anticoagulation is crucial. Third, how should anticoagulant-refractory ICoVT, as observed in our patient, be managed? The evidence and guidelines on these conditions are currently lacking.

Much can be learned from our failed treatments. For example, the aGAPSS can help identify patients at a high risk of thrombosis development and recurrence (7, 9). In the 2019 Chinese guideline for CVST, the therapeutic dosage of LMWH was 0.4–0.6 ml twice per day (13). However, the recommended dosage may not be sufficient for patients with APS and should be adjusted according to risk stratification and anticoagulation monitoring (7, 26). The aGAPSS, which is developed to quantify thrombosis risk, is calculated by adding together the corresponding points to risk factors: 5 for aCL (IgG/IgM), 4 for LA, 4 for aβ2GPI (IgG/IgM), 3 for dyslipidemia, and 1 for arterial hypertension (32). aGAPSS risk is stratified as high risk (≥ 12 points), medium risk (6–11 points), and low risk (<6 points) according to Radin's classification (33). Our case had triple positivity and an aGAPSS of 17 points, which resulted in the highest risk for venous thrombosis; moreover, she presented with gradual deterioration of neurological function despite receiving standard-dose LMWH. A reasonable approach is high-intensity LMWH at approximately 25% or 33% above the standard dose. The anti-Xa chromogenic assay is the best assay for monitoring LMWH (23); the recommended peak anti-Xa is 0.5–0.8 IU/ml for standard treatment and 0.8–1.0 IU/ml for high-intensity treatment (7, 23). In fact, both the initial and adjusted LMWH doses in our case appeared insufficient for achieving our treatment goals. Thus, we recommend that the LMWH dose be adjusted according to the anti-Xa chromogenic assay, especially in anticoagulant-refractory patients. Additionally, MRBTI can non-invasively and quantitatively assess the thrombus load, which is of significant value in the evaluation of anticoagulation effects. However, catastrophic APS requires careful consideration when thromboses occur in multiple organs. Aside from providing adequate anticoagulation and eliminating precipitating factors, early treatment is critical for catastrophic APS and includes corticosteroids, intravenous immunoglobulin, plasma exchange, B-cells inhibition, and complement inhibition (34). Awareness that direct-acting oral anticoagulants should be avoided in patients with APS is critical (9, 23). Vitamin K antagonist with a target INR of 2–3 is strongly recommended for long-term prevention (2, 13).

Although anticoagulation therapy remains the cornerstone of treatment to prevent thrombosis in primary APS patients, this treatment may be insufficient in more than 5% of cases (35, 36). Hydroxychloroquine, which was used in our case, has gradually become recognized as an effective adjuvant treatment for thrombosis in APS patients owing to its antithrombotic, anti-inflammatory, and immunomodulatory properties (35, 36). In a recent randomized prospective study involving 50 patients with primary APS, patients treated with hydroxychloroquine had a 91% lower risk of thrombosis than patients who received standard care alone (hazard ratio, 0.09; 95% confidence interval, 0.01–1.26, p = 0.074) (35). In addition, the administration of hydroxychloroquine can significantly reduce thrombus size and duration (36).

Several therapeutic options are available for APS-CVST patients who are refractory to anticoagulant therapy. Current evidence does not support the use of systemic thrombolysis before anticoagulant therapy. Ongoing anticoagulant therapy is a contraindication of systemic thrombolysis. Recent years have seen an increase in the use of endovascular therapy (EVT), including intrasinus thrombolysis (IST) and mechanical thrombectomy (MT), in patients who are refractory to anticoagulant therapy (37). Thrombolytic agents can be directly delivered to the site of the thrombosed venous to dissolve the thrombus. One study of 156 CVST patients treated with IST showed that functional independence was achieved in 91.38% of patients, whereas complete or partial recanalization was achieved in 93.96% of patients (38). The rate of major hemorrhagic complications has been reported to be 9.8%–10.7% across studies (37). MT is an alternative EVT method that offers higher rates of successful recanalization and a lower risk of hemorrhagic complications. Various techniques and devices have been applied to clinical practice with and without IST. A systematic review of 17 studies and 235 severe CVST patients showed that a favorable outcome is achieved in 76% of patients, and the mortality rate is 14.3% (39). Notably, 87.6% of patients underwent concomitant IST and MT (39). As mentioned above, IST and MT may be reasonably effective and safe in patients with a high risk of poor prognosis. Recently, a retrospective study reported that IST and MT are also effective and safe in most patients with CVST with ICH (40), which strongly reinforces the hypothesis that ICH is not a contraindication for IST or MT. However, these therapeutic options may not be applicable to ICoVT patients because of the distinctive histopathological characteristics of ICoVT.

Decompressive craniectomy (DC) is a well-established life-saving procedure and also the only treatment for malignant CVST or ICoVT (41). Because evidence regarding the benefits of DC was limited until 2011, the 2011 American Heart Association/American Stroke Association guideline only weakly recommended that DC "might be" needed as a life-saving measure (42). Recently, growing evidence indicates that DC not only improves survival but also achieves acceptable outcomes, even in critically ill patients. A systematic review of five studies published from 2012 to 2018 evaluated the role of DC in CVST. In the pooled analysis, the mortality rate was 16.1%, and favorable outcomes with an mRS score of 0–2 were achieved in 54.4% of patients (41). Thus, the ESO guidelines and the 2019 Chinese guidelines strongly recommend DC for patients with impending herniation (13, 43). Additionally, a recent retrospective study of 48 patients with malignant CVST who were divided into a DC group and a medical group (44) reported that the mortality and favorable outcome rates of the DC group were consistent with those of previous studies. However, none of the patients in the medical group survived during hospitalization, which indicates that patients with malignant CVST do not benefit from endovascular or medical treatment (44). The current guidelines do not elaborate on the indications for DC (13, 43). In the published literature, DC is recommended to be offered as early as possible in the presence of

clinical or radiological signs of impending or established herniation, such as third nerve palsy, progressive neurological deterioration with a decrease in Glasgow coma score of more than 4 points, the threat of cerebral herniation, midline shift of ≥ 5 mm, and obliteration of the basal cisterns (41, 44). The optimal timing for restarting anticoagulation has not yet been elucidated.

CONCLUSION

ICoVT is a rare yet potentially fatal manifestation of APS. Clinicians should consider ICoVT in patients who present with an increased D-dimer level and symptoms of focal neurologic deficit and/or seizures. APS should be considered in patients with ICoVT, particularly young women. Neuroimaging is the cornerstone of ICoVT diagnosis, and multi-parametric MRI is required to identify this rare entity. Prompt anticoagulation and close monitoring are essential to achieve good clinical outcomes in patients with APS-related ICoVT.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Deidentified data, including clinical manifestations, neuroimaging data, serum tests, and cerebrospinal fluid tests, are available upon appropriate request to the corresponding author.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

FF and YL contributed to the design of the study, revised the manuscript, and were responsible for the integrity and accuracy of the data. JShen contributed to drafting the manuscript and reviewing the published literature. ZT contributed to the collection of clinical and neuroimaging data. JSun was the attending doctor of the patient and contributed to the acquisition and analysis of the clinical data. WC contributed to the analysis of the neuroimaging data. All authors contributed to the article and approved the submitted version.

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Anti-Phospholipid Antibodies and Coronavirus Disease 2019: Vaccination Does Not Trigger Early Autoantibody Production in Healthcare Workers

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A molecular mimicry between severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and human proteins supports the possibility that autoimmunity takes place during coronavirus disease 2019 (COVID-19) contributing to tissue damage. For example, anti-phospholipid antibodies (aPL) have been reported in COVID-19 as a result of such mimicry and thought to contribute to the immunothrombosis characteristic of the disease. Consistently, active immunization with the virus spike protein may elicit the production of cross-reactive autoantibodies, including aPL. We prospectively looked at the aPL production in healthcare workers vaccinated with RNA- (BNT162b2, n. 100) or adenovirus-based vaccines (ChAdOx1, n. 50). Anti-cardiolipin, anti-beta2 glycoprotein I, anti-phosphatidylserine/prothrombin immunoglobulin G (IgG), IgA, and IgM before and after vaccination were investigated. Anti-platelet factor 4 immunoglobulins were also investigated as autoantibodies associated with COVID-19 vaccination. Additional organ (anti-thyroid) and non-organ (anti-nuclear) autoantibodies and IgG against human proteome were tested as further post-vaccination autoimmunity markers. The antibodies were tested one or three months after the first injection of ChAdOx1 and BNT162b2, respectively; a 12-month clinical follow-up was also performed. Vaccination occasionally induced low titers of aPL and other autoantibodies but did not affect the titer of pre-existing autoantibodies. No significant reactivities against a microarray of approximately 20,000 human proteins were found in a subgroup of ChAdOx1-

vaccinees. Consistently, we did not record any clinical manifestation theoretically associated with an underlying autoimmune disorder. The data obtained after the vaccination (two doses for the RNA-based and one dose for the adenovirus-based vaccines), and the clinical follow-up are not supporting the occurrence of an early autoimmune response in this cohort of healthcare workers.

Keywords: anti-phospholipid antibodies, SARS-CoV-2 vaccination, autoimmunity, autoantibodies, COVID-19

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and, in particular, the Spike protein share common amino acid linear sequences and some conformational structures with human proteins (1, 2). Consistently, an autoimmune signature was suggested to take place in Coronavirus disease 2019 (COVID-19) because of the molecular mimicry phenomenon and the deregulated immune response caused by the virus itself (3–8). Autoantibodies against self-antigens, in particular, anti-phospholipid antibodies (aPL), were reported in COVID-19 patients and their pathogenic role in the disease was discussed (9–12).

If the molecular mimicry with the virus, and in particular with the Spike protein, is playing a role in COVID-19, then the SARS-CoV-2 vaccines could potentially trigger autoimmune responses as well. Consistent with this hypothesis are the induction of the vaccine-induced thrombocytopenic thrombotic (VITT) syndrome and anecdotal cases of *de novo* autoimmune diseases triggered by the anti-SARS-CoV-2 vaccination (13–15). However, the occurrence of autoantibodies before and after vaccination in healthy subjects has not been investigated, and very few studies have addressed whether the vaccines affect autoantibodies already present in autoimmune patients (16, 17). On the other hand, this aspect is becoming of paramount importance because of the large number of vaccinated people and the need for repeated booster injections.

With this in mind, we planned a prospective study to look at the production of autoimmune antibodies in healthcare workers vaccinated with RNA- or adenovirus-based vaccines. Anti-phospholipid antibodies and additional organ and non-organ specific autoantibodies were tested before and after the vaccination. To further investigate whether active immunization may trigger autoimmune responses, the autoantibodies against an array of human proteins before and after vaccination were also investigated in a subgroup of ChAdOx1-vaccinees and the clinical follow-up was planned for all the vaccinated subjects up to 12 months after the first dose of the vaccines. The data obtained after the vaccination (two doses for the RNA-based and one dose for the adenovirus-based vaccines) and the clinical follow-up are apparently ruling out the occurrence of early autoimmune response in this cohort of healthcare workers.

MATERIALS AND METHODS

Vaccinated Subjects

One hundred healthcare workers vaccinated with BNT162b2 (Comirnaty) and 50 with ChAdOx1 (Vaxzevria) between January and February 2021 were included in the study. The inclusion criteria were age >18 years and availability for serial blood samples collection. The exclusion criteria were pregnancy, breastfeeding, autoimmune diseases, other vaccinations in the past 6 months (with the exception of the flu vaccination), and severe adverse effects to previous vaccinations. The demographic characteristics of the vaccinated subjects are reported in **Supplementary Table 1**. Two subjects, vaccinated with BNT162b2, were known to have subclinical autoimmune thyroiditis without any treatment. Fifteen of 100 subjects vaccinated with BNT162b2 and five of 50 subjects vaccinated with ChAdOx1 had mild or asymptomatic COVID-19 confirmed by means of a positive reverse transcription polymerase chain reaction (RT-PCR) for SARS-CoV-2. The infections were documented before the vaccination.

Adverse side effects as defined by Polack et al. (18) or any clinical manifestation potentially correlated with vaccination were also recorded for all the investigated subjects (**Supplementary Table 2**). The follow-up of the vaccinated subjects was carried out for 12 months starting from the first injection of the vaccine.

The Ethics Committee at Istituto Auxologico Italiano approved the study (08–01–2021).

Vaxzevria samples were collected at IRCCS Ca' Granda Ospedale Maggiore Policlinico in Milan (Polimmune-COVID: approved by Institutional Review Board Milano Area 2 and by the National Ethical Committee of Ospedale Spallanzani, #331_2020 and #665_2020). All the subjects gave their informed consent and their privacy rights are observed.

Anti–Severe Acute Respiratory Syndrome Coronavirus 2 Serological Profile

The presence of total Ig against the SARS-CoV-2 nucleocapsid and the spike protein RBD was detected by a chemiluminescence solid-phase assay according to the manufacturer's instructions (Roche, Milan, Italy). Values ≥ 0.80 U/ml were considered positive for anti-RBD Ig; anti-N Ig levels were expressed as a cutoff index (ICO ≥ 1).

Anti–SARS-CoV-2 antibodies were detected immediately before vaccine administration (T0) and 1 month or 3 months after the first injection of ChAdOx1 or BNT162b2, respectively (one dose for the adenovirus-based and two doses for the RNA-vaccine) (T1).

Detection of Diagnostic Autoantibodies

An autoantibody profile including both organ- and non-organ specific antibodies was investigated in serum samples collected at T0 and T1 as stated above.

Anti-cardiolipin (aCL) and anti-beta2 glycoprotein I (β_2GPI) immunoglobulin G (IgG), IgM, and IgA were measured by in-house enzyme-linked immunosorbent assay (ELISA), as previously described (17); anti-phosphatidylserine/prothrombin (PS/PT) IgG and IgM were detected by commercial ELISA (Inova Diagnostics, S. Diego, CA, USA), according to the manufacturer's protocol.

Antinuclear IgG antibodies (ANA) were detected by a commercial chemiluminescence solid-phase assay (CTD screen PLUS; QUANTA Flash, Inova Diagnostics, San Diego, CA, USA) that detects the following cell autoantigens: dsDNA, RNP, Sm, Scl-70, Jo-1, Ro52, Ro60, SS-B, Centromere, RNA Pol III, Pm/Scl, Mi-2, PCNA, Th/To, Ku, ribosomal-P. The assay was performed according to the manufacturer's instructions (19). The use of a solid-phase assay for ANA as a screening assay for systemic lupus erythematosus classification and for the diagnosis of other ANA-associated rheumatic diseases has been recently suggested and supported by recommendations of international scientific societies (20–22).

Anti-platelet factor (PF) 4 antibodies IgG/IgA/IgM were tested by ELISA (Immucor, Solihull, UK), as reported (17).

Antibodies against thyroid-stimulating hormone (TSH) receptor (TSH-R), thyroglobulin (TG), and thyroid myeloperoxidase (TPO) were detected by a commercial chemiluminescence solid-phase assay (Phadia AB, Uppsala, Sweden) and expressed in IU/ml (TG, TPO) or IU/l (TSH-R), according to the manufacturer's instructions.

HuProt Arrays and Serum Profiling Assays

The human proteome microarrays (HuProt) arrays were manufactured by CDI LABS (CDI Laboratories, Inc., Mayaguez, PR, USA). Each HuProt array v4.0 consists of >20,000 human proteins, most full length, and representing >16,000 human protein-coding genes, covering about 80% of the human proteome. The arrays also contained IgG spotted at different concentration (100, 25, 6.25, and 1.56 ng/µl). Incubation was performed according to the manufacturer's instructions. Briefly, the arrays were blocked using blocking buffer (2% BSA in phosphate-buffered saline [PBS] buffer with 0.05% Tween 20) at 4°C overnight. Then, each serum sample was diluted 1:500 in blocking buffer and incubated at room temperature (RT) for 2h with gentle rocking. After 3 × 10 min washes with TPBS (PBS buffer with 0.05% Tween 20), the arrays were incubated for 2h at RT with Alexa 647–conjugated goat anti-human IgG (Thermo Fisher Scientific, Walthman, MA, USA) (1:800 in blocking buffer) in the dark with gentle rocking. The arrays were then washed twice in TPBS, twice in 0.1× PBS, and finally once in milliQ sterile water. The slides were finally dried at 30°C under nitrogen and scanned using a ScanArray Gx PLUS (Perkin Elmer, Shelton, CT, USA). Images (16 bit) were generated with the ScanArray™ software at a resolution of 10 µm per pixel and analyzed using ImaGene 9.0 software (Biodiscovery Inc., Hawthorne, CA, USA). A 635-nm laser was used to excite the Alexa-647 dye.

HuProt arrays were used to analyze the reactivity of sera from 10 individuals collected pre and post-ChAdOx1 vaccination, five additional individuals only post-ChAdOx1vaccination, and 10 age and sex-matched blood donors (healthy controls, HC sera) collected before the COVID-19 pandemic and retrieved from the archives of IRCCS Istituto Auxologico Italiano and Ospedale Maggiore Policlinico, Transfusional Unit, Milan, Italy. These HC samples were tested on the proteome array in order to identify occasional reactivities spontaneously arising in healthy individuals.

HuProt Arrays Data Analysis

For each sample, the raw mean fluorescence intensity (MFI) values of each spot were measured, signal-to-local-background ratios were calculated using ImaGene, and spot morphology and deviation from the expected spot position were considered using the default ImaGene settings. For each sample, the background-subtracted MFI of replicated spots was determined. For each protein, a coefficient of variation (CV%) was calculated on replicate spots, for intra-assay reproducibility. Each protein was checked for displaying a CV% correlated to its MFI on the basis of standard IgG curves. If the CV% value was not within the expected range the protein was not considered for further analysis. In order to compare the autoreactivities from independent experiments, the MFI values were normalized based on the IgG curve spotted on each slide (**Supplementary Figure 1**). On the basis of this analysis, we also derived the concentration of autoantigen-specific IgG in ng/µl (**Supplementary Figure 1**). Autoantigens were identified using two different comparisons: (1) 15 post-vaccination versus 10 pre-vaccination sera, using as threshold a fold change of MFI values > 2.5; 2) 15 post-vaccination versus 10 HC samples. In the latter analysis, we selected autoantigens matching these multi-criteria: (i) not recognized by any of HC sera, (ii) having fold change > 2.5 in at least one vaccinated individual, and (iii) having concentration >30 ng/µl (this concentration threshold was established based on the protein reactivity distribution of ChAdOx1 towards HC sera, as shown in **Supplementary Figure 3**).

Statistical Analysis

The demographic and clinical characteristics of participants were summarized using descriptive statistics. Normalized data and statistical analysis of proteins spotted into the HuProt Array were analyzed by R. Differential analysis between pre-vaccine and post-vaccine were performed with Limma package and *p*-values were corrected by Benjamini-Hochberg procedure.

RESULTS

Response to Vaccination of Healthcare Workers

All the vaccinated subjects displayed Ig values against the SARS-CoV-2 spike protein RBD higher than the established cutoff. Twenty of 150 subjects reported previous history of SARS-CoV-2 infection and displayed Ig against the virus nucleocapsid as well.

The percentages of non-severe side effects after the vaccinations were comparable to those reported in the

literature (18) and are shown in **Supplementary Table 2**; no severe side effects were reported.

All the enrolled subjects have been followed up for 12 months starting from the first injection of the two vaccines.

Identification of a Panel of Diagnostic Autoantibodies in Vaccinated Healthcare Workers

Since aPL and anti-PF4 antibodies were reported in few COVID-19 patients (12, 17) and anti-PF4 antibodies have been associated with the development of VITT after ChAdOx1 vaccination (13–15), we detected the abovementioned autoantibodies in our cohort of vaccinated healthcare workers. Anti-nuclear antibodies were also occasionally reported, and there is evidence of thyroid involvement in COVID-19 patients (9, 23, 24). In keeping with this finding, we tested our vaccinated subjects for an ANA screening assay and anti–TSH-R, anti-TG, and anti-TPO antibodies as further clues of an autoimmune signature. As shown in the heatmaps of **Figure 1**, only occasional positivity was found and the percentages of positivity were very small or the search resulted negative. In particular, we did not detect any new autoantibody positivity or increased autoantibody titers after both vaccines. The positive results were found at low titer with weak clinical diagnostic/prognostic value. This was particularly true for IgA anti-β2GPI positivity (25, 26).

Identification of Autoantigens Recognized by Sera of Subjects Pre- and Post-Vaccination

An important question is whether vaccination may alter autoreactivity in healthy individuals. Keeping this in mind, we evaluated the autoantigen reactivity of sera collected from 15 individuals who received ChAdOx1 against a broad spectrum of human proteins. For 10 of the 15 individuals, we tested both the pre-vaccination and the post-vaccination serum samples. For the five remaining individuals, only the post-vaccination sample was used. The overall autoreactivity pattern of ChAdOx1 vaccinées toward self-proteins, based on the distribution profile of the MFI values for each protein represented on the array, was comparable to that of pre-vaccine and of non-vaccinated HC sera collected in the pre-COVID-19 period (**Figure 2**). None of the antigens showed a significant autoreactivity profile in ChAdOx1 compared to HC group (**Supplementary Figure 2**).

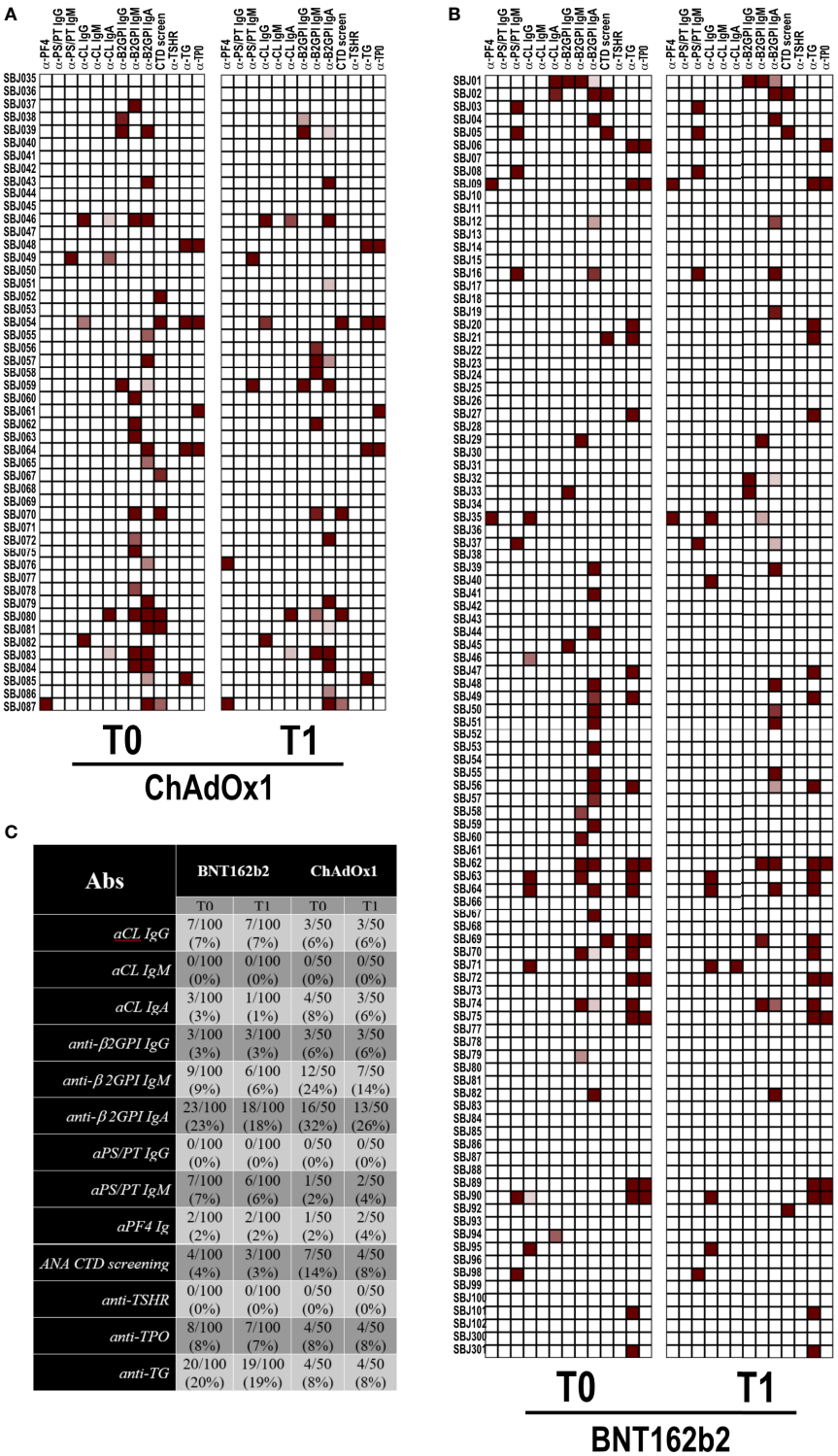
Volcano-plot analysis revealed only two highly reactive autoantigens (**Figure 3**), namely, Quinoid Dihydropteridine Reductase (QDPR) and Poly(rC)-binding protein 3 (PCBP3). Frequency analysis indicated that QDPR showed the highest autoreactivity, being recognized by all the subjects (including the HC), whereas PCBP3 was recognized only by one subject after vaccination.

Then, we further investigated specific autoantibodies arising with low frequency upon ChAdOx1 vaccination as detailed in the paragraph 2.5, Materials and Methods section. In this way, we excluded occasional reactivities spontaneously arising in healthy individuals, not specifically ascribable to COVID-19, and we identified 59 proteins, showing a scattered immune-reactivity among the vaccinated individuals (**Figure 4** and **Supplementary Table 3**).

Overall, our analysis showed that ChAdOx1 vaccination does not alter the autoreactivity of vaccinated individuals and only sporadically induces autoantibodies. The subjects who resulted positive for these antibodies have been asymptomatic for the whole clinical follow-up.

DISCUSSION

Molecular mimicry between the virus and self-antigens was suggested to trigger the production of autoantibodies in COVID-19 and potentially after SARS-CoV-2 vaccination as well. A lot of attention has been paid to aPL in COVID-19 as a prototype of autoantibodies potentially contributing to the thrombophilic state that characterizes the most severe forms of COVID-19. So, we firstly investigated whether the active immunization with both RNA- and adenovirus-based vaccines against SARS-CoV-2 induces the production of aPL as markers of a pro-thrombotic phenotype in asymptomatic healthcare workers. We also investigated anti-PF4 Ig as autoantibody markers of the thrombotic thrombocytopenic purpura that has been recently described after the adenovirus-based anti–SARS-CoV-2 vaccine. Additional non-organ specific, such as ANA, and organ-specific anti-thyroid autoantibodies were evaluated before and after the vaccination, since all of them have been reported in COVID-19 as markers of an unwanted autoimmune response. Our results support the safety of the vaccination from this point of view, since all these autoantibodies have not been developed early after the first injection. Moreover, there is no evidence that the vaccination may affect the titer of pre-existing autoantibodies occasionally detectable in the asymptomatic subjects before the vaccination. In particular, this is true for aPL in agreement with similar data reported in patients suffering from the anti-phospholipid syndrome that received the vaccination against SARS-CoV-2 (17). Consistently, we did not record any clinical manifestation theoretically associated with the development of an underlying autoimmune disorder. This finding is well supported by the data from the vaccinated subjects investigated before and after the complete vaccination with BNT162b2 (two doses). Although the subjects that received ChAdOx1 were tested before and after the first dose only, the absence of newly developed autoantibodies or of clinical manifestations 1 month after the first vaccine injection may support the same conclusion. In fact, the best described autoimmune adverse reaction after ChAdOx1 is the VITT associated with the appearance of anti-PF4 Ig usually a few days after the first administration of ChAdOx1. This suggests that a single immunization can be sufficient for triggering an autoimmune response and the clinical syndrome (13–15). Although some of these autoantibodies resulted positive at low titer, the lack of titer modification before and after the vaccination and the absence of clinical or laboratory signs of autoimmune disorders do support the occurrence of the so-called "innocent" antibodies, as we recently described (12, 17).



C

Abs	BNT162b2		ChAdOx1	
	T0	T1	T0	T1
aCL IgG	7/100 (7%)	7/100 (7%)	3/50 (6%)	3/50 (6%)
aCL IgM	0/100 (0%)	0/100 (0%)	0/50 (0%)	0/50 (0%)
aCL IgA	3/100 (3%)	1/100 (1%)	4/50 (8%)	3/50 (6%)
anti-β2GPI IgG	3/100 (3%)	3/100 (3%)	3/50 (6%)	3/50 (6%)
anti-β 2GPI IgM	9/100 (9%)	6/100 (6%)	12/50 (24%)	7/50 (14%)
anti-β 2GPI IgA	23/100 (23%)	18/100 (18%)	16/50 (32%)	13/50 (26%)
aPS/PT IgG	0/100 (0%)	0/100 (0%)	0/50 (0%)	0/50 (0%)
aPS/PT IgM	7/100 (7%)	6/100 (6%)	1/50 (2%)	2/50 (4%)
aPF4 Ig	2/100 (2%)	2/100 (2%)	1/50 (2%)	2/50 (4%)
ANA CTD screening	4/100 (4%)	3/100 (3%)	7/50 (14%)	4/50 (8%)
anti-TSHR	0/100 (0%)	0/100 (0%)	0/50 (0%)	0/50 (0%)
anti-TPO	8/100 (8%)	7/100 (7%)	4/50 (8%)	4/50 (8%)
anti-TG	20/100 (20%)	19/100 (19%)	4/50 (8%)	4/50 (8%)

FIGURE 1 | Autoantibody reactivity pre- and post-vaccination by BNT162b2 or ChAdOX1. Heatmap showing serum IgG reactivity of individuals vaccinated with **(A)** ChAdOX1 or **(B)** BNT162b2 to the indicated autoantigens. In red are depicted the reactive subjects (arranged in rows) for each autoantigen (in column). **(C)** Frequencies of reactivity for each autoantigen.

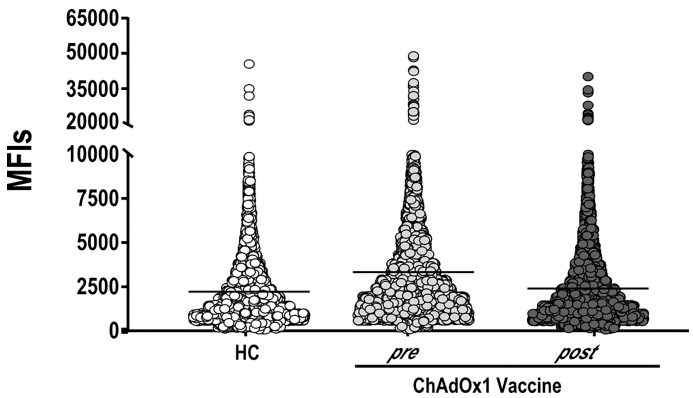


FIGURE 2 | ChAdOx1 vaccination does not alter the general autoimmunoreactivity. Mean Fluorescence Intensities values (MFIs) of all proteins spotted on the HuProt array probed with sera of ChAdOx1 individuals at pre- (n = 10) and post-vaccination (n = 15) and 10 Healthy Controls (HC). Each dot represents the MFI of a single protein within the groups of sera reported on the x-axis.

We also found few reactivities of the sera from the same cohort against a human protein microarray representing approximately 20,000 human proteins. Since adenovirus-based vaccines has the highest prevalence of severe adverse events associated with definitive autoantibody immunity compared to mRNA vaccines (13–15), we investigated the autoreactivity against the proteome microarray in subjects that received the ChAdOx1 vaccine only. We did not observe any significant alteration of the overall reactivity before and after vaccination or comparing the vaccinated subjects to the non-vaccinated pre-Covid-19 HCs. Indeed, PCBP3 and QDPR 1 were the only two antigens against which we found a high reactivity. However, PCBP3 was recognized by only one subject post-vaccination, whereas QDPR was recognized by all the individuals, including the healthy donors, thereby unlikely being specifically induced by the ChAdOx1 vaccine. In addition, when comparing ChAdOx1 vaccinated versus non-vaccinated healthy individuals, we identified 59 proteins that were recognized only sporadically and with low frequencies after vaccination, thus not allowing the definition of a vaccine-related reactivity pattern.

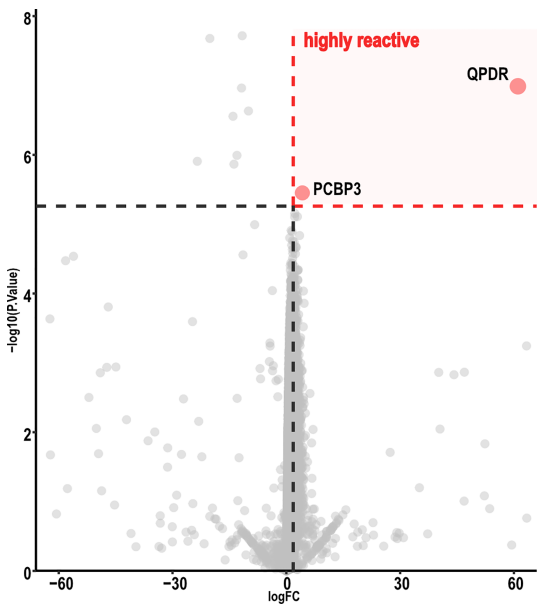


FIGURE 3 | Volcano map analysis of ChAdOx1 samples. Volcano plot representing FC versus P-values of each protein displayed only two proteins highly reactive with ChAdOx1 serum samples. Graphic representation focuses from −60 to 60 in a log FC range. Thresholds were established by analysis of the FC (≥ 2.5) and adjusted P-value (< 0.01). ChAdOx1 was compared by R package Limma; Benjamini, as well as Hochberg procedure, was used to adjust P-values.

We did not test the samples after BNT162b2 administration, since autoantibody-mediated side-effects have been less frequently reported in RNA-based vaccines (27, 28).

Our data support the view that the immunization with the SARS-CoV-2 spike protein may trigger the production of low titer of aPL and other autoantibodies but does not affect the titer of pre-existing autoantibodies detectable in asymptomatic healthcare workers, ruling out the possibility of a widespread autoimmune humoral response shortly after vaccination.

On the other hand, there is evidence that autoimmune responses can take place in patients with acute COVID-19. In particular, autoantibodies were described against lung endothelial and epithelial cell surface antigens in patients suffering from SARS-CoV-1 disease (29). More recently, significant deposition of Ig and complement has been described in post-mortem biopsy specimens of various organs collected from COVID-19 patients but not in SARS-CoV-2 negative pathological controls (30). The lack of co-localization of the spike protein with the Ig and complement deposits was suggested to be the consequence of an auto-reactivity against self-antigens presented by the damaged tissues (30). Consistent with this hypothesis, two groups reported the new onset of autoantibodies against self-proteins in hospitalized COVID-19

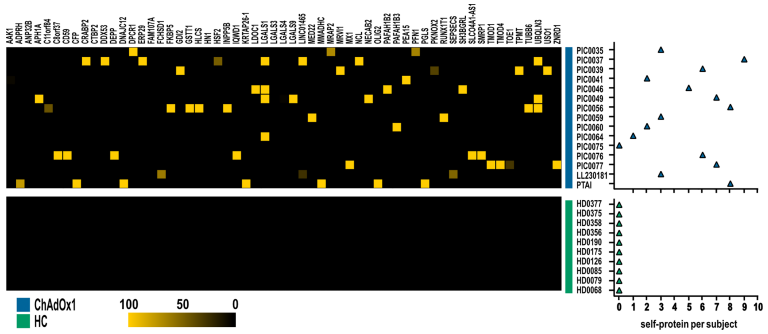


FIGURE 4 | Autoantigens specifically recognized by at least one subject in ChAdOx1 group as opposed to HC. Heatmap depicting IgG reactivities of ChAdOx1vaccinated subject (*n* = 15, in blue) collected post-vaccination, and healthy donors (HC; *n* = 10, in green), arranged in rows, for the 59 antigens (in column). Yellow indicates positive immune reactivity (>30 ng/μl), and black indicates low or no immune-reactivity (<30 ng/μl). The count of the self-proteins recognized by ChAdOx1 vaccinated subjects and HC (blue and green, respectively) is shown in the right panel. Counts were based on antibodies that were present at levels ≥30 ng/μl.

patients and in healthcare workers with mild or asymptomatic infection (31, 32). It has been suggested that these autoantibodies may play a role in triggering and/or supporting the clinical manifestations of the disease. The lack of autoreactivity early after vaccination is not in clash with the abovementioned data, since active immunization is not associated with extensive inflammation and tissue damage as in the COVID-19, and the presentation of self-antigens in immunogenic forms is not likely to take place in the vaccinated subjects.

Autoimmune diseases are characterized by a long incubation period and the associated autoantibodies may be detectable even years before the clinical manifestations. We cannot rule out that autoimmune markers or even clinical autoimmune manifestations can develop over time, and the study has been planned to collect data and further biological samples in a longer follow-up (more than one year) taking advantage of the presence of the vaccinated subjects in our institutions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee at Istituto Auxologico Italiano (08-01-2021). Vaxzevria samples were collected at IRCCS Ca' Granda Ospedale Maggiore Policlinico in Milan (Polimmune-COVID: approved by Institutional Review Board Milano Area 2 and by the National Ethical Committee of Ospedale Spallanzani, #331_2020 and #665_2020). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MOB, MB, RG, and PLM contributed to conception and design of the study and wrote the first draft of the manuscript. CB, PL, AGob, and ML performed the assays for autoantibodies detection, collected the results, and organized the database. ET, AD, IB, and FS provided the clinical data. AB and AGor evaluated the serological response to the vaccines. MB, AGob and FP performed the statistical analysis. GP and SA supervised the study. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.930074/full#supplementary-material

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A novel aGAPSS-based nomogram for the prediction of ischemic stroke in patients with antiphospholipid syndrome

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Background: Ischemic stroke (IS) is the most common and life-threatening arterial manifestation of antiphospholipid syndrome (APS). It is related to high mortality and severe permanent disability in survivors. Thus, it is essential to identify patients with APS at high risk of IS and adopt individual-level preventive measures. This study was conducted to identify risk factors for IS in patients with APS and to develop a nomogram specifically for IS prediction in these patients by combining the adjusted Global Anti-Phospholipid Syndrome Score (aGAPSS) with additional clinical and laboratory data.

Methods: A total of 478 consecutive patients with APS were enrolled retrospectively. All patients were randomly assigned to the training and validation cohorts. Univariate and multivariate binary logistic analyses were conducted to identify predictors of IS in the training cohort. Then, a nomogram was developed based on these predictors. The predictive performance of the nomogram for the training and validation cohorts was evaluated by determining areas under the receiver operating characteristic curve (AUROC) and creating calibration plots. A decision curve analysis (DCA) was conducted to compare the potential net benefits of the nomogram with those of the aGAPSS.

Results: During a mean follow-up period of 2.7 years, 26.9% (129/478) of the patients were diagnosed with IS. Binary logistic regression analysis revealed that five risk factors were independent clinical predictors of IS: age ($P < 0.001$), diabetes ($P = 0.030$), hyperuricemia ($P < 0.001$), the platelet count ($P = 0.001$), and the aGAPSS ($P = 0.001$). These predictors were incorporated into the nomogram, named the aGAPSS-IS. The nomogram showed satisfactory performance in the training [AUROC = 0.853 (95% CI, 0.802–0.896] and validation [AUROC = 0.793 (95% CI, 0.737–0.843)] cohorts. Calibration curves showed good concordance between observed and nomogram-predicted probability in the training and validation cohorts. The DCA confirmed that the aGAPSS-IS provided more net benefits than the aGAPSS in both cohorts.

Conclusion: Age, diabetes, hyperuricemia, the platelet count, and the aGAPSS were risk factors for IS in patients with APS. The aGAPSS-IS may be a good tool for IS risk stratification for patients with APS based on routinely available data.

KEYWORDS

antiphospholipid syndrome, adjusted Global Anti-Phospholipid Syndrome Score, ischemic stroke, nomogram, risk stratification

Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by recurrent thrombotic events and pregnancy morbidity associated with the persistent presence of antiphospholipid antibodies (aPLs) (1). Ischemic stroke (IS) is one of the most common central nervous system manifestations and the most life-threatening complication of APS (2, 3). It accounts for nearly half of the arterial events caused by APS (4). Nearly 20% of cerebral strokes in patients younger than 50 years have been suggested to be associated with APS (5). In addition, cerebral infarction was reported in 19.8% of a cohort of 1000 European patients with APS and accounted for 11.8% of all deaths that occurred during a 10-years follow-up period (6). IS is associated with a high mortality rate and severe permanent disability in survivors (7). In 2019, stroke led to 6.55 million deaths and 143 million disability-adjusted life years on average, 62.4% of which were ischemic strokes (8). Thus, it is essential to identify patients with APS at high risk of IS and adopt individual-level preventive measures.

No widely accepted predictive tool or model has been established for aPL-positive patients. The Global Antiphospholipid Syndrome Score (GAPSS) and antiphospholipid score (aPL-S) are used to predict thrombosis in patients with APS (9, 10). Although their utility has been validated with various external cohorts, they rely on aPL quantification (11–13), which is difficult due to the problems with the standardization of aPL criteria and the difficulty of interpreting lupus anticoagulant results (14). Furthermore, these scoring systems require data on laboratory parameters not routinely measured in daily clinical practice. GAPSS modifications, including the adjusted Global Antiphospholipid Syndrome Score (aGAPSS) and aGAPSS specific for cardiovascular disease ($aGAPSS_{CVD}$), have been proposed (15, 16), and patients with primary APS who had experienced IS were found to have higher aGAPSS (17). However, little is known about the efficiency of the aGAPSS for the prediction of IS in patients with APS. Traditional thrombotic risk factors, including obesity, smoking habit, and diabetes, also increase the risk of thrombosis in these patients (18).

In the present study, we evaluated the risk factors for IS in patients with APS. We also developed a new nomogram specifically for IS prediction in these patients by combining the aGAPSS with additional risk factors.

Methods

Patients and baseline data collection

Consecutive patients with APS who attended Peking University People's Hospital between 1 January 2005 and 1 March 2021 were enrolled retrospectively in this study. All participants met the 2006 Sydney classification criteria for APS (1). The exclusion criteria were: 1) IS occurrence before APS onset; 2) other coagulation disorders, such as severe hepatic diseases and malignancy; and 3) incomplete medical records. The following clinical data were collected at the time of APS diagnosis: age, sex, body mass index, time from first APS event, history of autoimmune disease (e.g., systemic lupus erythematosus, Sjögren's syndrome), vascular thrombosis, pregnancy morbidity, hypertension, hyperlipidemia, diabetes, chronic obstructive pulmonary disease (COPD), chronic kidney disease, hyperuricemia, smoking, laboratory data, and treatment. Patients were followed by telephone interviews or clinic visits every three months.

Assessment of risk factors for ischemic stroke

According to the guidelines of the American Stroke Association (12), hypertension, diabetes, smoking, and hyperlipidemia were considered to be traditional risk factors for IS. Hypertension, diabetes, and smoking were assessed according to the guidelines of the National Institute for Health and Care Excellence (19). Hypertension was defined as high blood pressure at two or more random time points or the use of antihypertensive medication. Diabetes was defined as a fasting blood glucose level > 7.0 mmol/L on more than two occasions or the use of insulin or oral antidiabetic

drugs. Smoking status was determined by self-reports of tobacco consumption. According to the Chinese Guideline for the Management of Dyslipidemia in Adults (20), hyperlipidemia was defined by any of the following criteria: 1) triglyceride level > 2.3 mmol/L, 2) high-density lipoprotein level < 1.0 mmol/L, 3) low-density lipoprotein level > 4.1 mmol/L, and 4) total cholesterol level > 6.2 mmol/L. Hyperuricemia was established when fasting serum urate levels equaled to or exceeded 420 μmol/L (21). Thrombocytopenia was defined as a platelet count $<100 \times 10^9$/L (1). In addition, diagnoses of COPD and chronic kidney disease, recently accepted as risk factors for stroke (22–24), were confirmed by medical record review.

Antiphospholipid antibodies detection and aGAPSS

Anti-cardiolipin (aCL) and anti-β2-glycoprotein I antibody (aβ2GPI) were detected by enzyme-linked immunosorbent assay as described previously (25). Values for aCL > 12 IU/mL and aβ2GPI > 27 RU/mL were considered positive based on local cut-off. The lupus anticoagulant (LAC) assay was performed using Stago STA Compact Hemostasis System as described previously (25). The simplified Dilute Russell's Viper Venom Test (dRVVT) was considered positive if the dRVVT ratios were > 1.2.

The aGAPSS was calculated as previously reported by adding corresponding points to the risk factors: 3 for hyperlipidemia, 1 for arterial hypertension, 5 for aCL, 4 for aβ2GPI, and 4 for LAC (10).

Assessment of ischemic stroke

Two experienced neurologists assigned patients to IS and non-IS groups based on clinical manifestations and neuroimaging (magnetic resonance imaging or computed tomography) findings. The diagnostic criteria for ischemic stroke are as follows: (1) acute onset; (2) focal neurological deficit (weakness or numbness of one side of the face or limb, speech impairment, etc.); (3) presence of a responsible lesion on imaging or signs/symptoms lasting more than 24 h; (4) exclusion of non-vascular causes; and (5) exclusion of cerebral hemorrhage by neuroimaging (26). Any inconsistency in the definition of IS was resolved by a senior neurologist.

Statistical analysis

The statistical analyses were performed using IBM SPSS Statistics (version 22.0), MedCalc software (version 20.1.0), and R software (version 4.1.2). By using a computer random number generator, one-half of the patients were randomized into the training cohort to construct the predictive nomogram, and the remaining patients were assigned to the validation cohort to evaluate the performance of the nomogram. Group comparisons were performed using the unpaired *t*-test (for normally distributed data) and Mann–Whitney *U* test (for non-normally distributed data) for quantitative variables, and Fisher's exact test and the chi-squared test for categorical variables. Receiver operating characteristic (ROC) curve analysis was used to determine the cutoff aGAPSS for discrimination of the IS and non-IS groups.

For the training cohort, univariate logistic regression analysis was performed to screen for potential predictors of IS. To identify independent risk factors for IS, variables with *P* values < 0.05 in the univariate analysis were included in a multivariate regression model based on the training cohort. The variance inflation factor (VIF) was used to measure the impact of collinearity among the variables in the regression model. Then, a nomogram was built based on these independent predictors using the rms package of the R software. The area under the receiver operating characteristic curve (AUROC) was drawn to evaluate and compare the discrimination efficacy of the nomogram with that of the aGAPSS. To access the predictive accuracy of the nomogram, calibration curves were drawn by plotting the observed probability against the nomogram-predicted probability. Finally, a decision curve analysis (DCA) was conducted with the rmda package to evaluate and compare potential net benefits at different threshold probabilities. For all statistical tests, two-sided *P* values < 0.05 were significant.

Results

Baseline clinical characteristics of study cohort

Of 505 patients with APS initially identified, three whose IS preceded APS onset, six with other coagulation diseases (four with malignancies and two with severe hepatic disease), and 18 whose medical records were incomplete were excluded. The remaining 478 patients were assigned randomly to the training and validation cohorts (n = 239 each; Figure 1). Baseline clinical data did not differ between cohorts (Table 1). During a mean follow-up period of 2.7 years, 129 (26.9%) patients were diagnosed with IS. Among all the IS patients in our study, eight patients had no definite neurological deficit symptom. Their silent ischemic lesions were identified through MRI scan due to nonspecific symptom (e.g., headache, dizziness), which showed high signal on the diffusion-weighted image (DWI). The DWI sequence has high accuracy for diagnosing IS (88%–100% sensitivity and 95%–100% specificity) (27). The IS incidence rates were similar in the training and validation cohorts [n = 69 (28.9%) and n = 61 (25.5%), respectively].

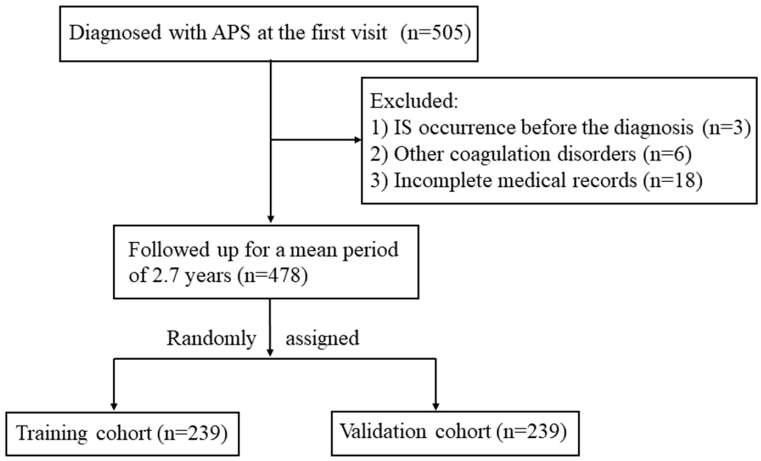

FIGURE 1
Study flow diagram. IS, ischemic stroke; APS, antiphospholipid antibody syndrome.

Construction of the predictive nomogram for IS

In the training cohort, patients with IS were older than those without IS [54.0 (43.0–64.0) *vs.* 37.0 (30.0–50.0) years, $P < 0.01$] and the proportion of males was larger in the former group (36.2% *vs.* 21.2%, $P = 0.02$). aGAPSS were higher for patients with than for those without IS [13.0 (11.0–16.0) *vs.* 9.0 (5.3–13.0), $P < 0.01$]. Greater prevalence of hypertension (50.7% *vs.* 24.1%, $P < 0.01$), diabetes (30.4% *vs.* 7.1%, $P < 0.01$), hyperlipidemia (65.2% *vs.* 50%, $P = 0.03$), and hyperuricemia (26.1% *vs.* 4.7%, $P < 0.01$) were also observed in patients with IS than in those without IS. Among laboratory parameters, the platelet count [114.9 (63.0–172.0) *vs.* 180.1 (104.3–237.5) $\times 10^9$/L, $P < 0.01$] was lower in the IS than in the non-IS group. The rate of aCL positivity (73.9% *vs.* 58.8%, $P = 0.03$), LAC positivity (79.7% *vs.* 53.5%, $P < 0.01$), and triple aPL positivity (44.9% *vs.* 25.9%, $P < 0.01$), were also higher in the IS than in the non-IS group (Table 2).

In the univariate analysis, IS was associated with age ($P < 0.001$), sex ($P = 0.017$), diabetes ($P < 0.001$), hyperuricemia ($P < 0.001$), autoimmune disease ($P = 0.020$), the platelet count ($P < 0.001$), and the aGAPSS ($P < 0.001$; Table 2). For the training cohort, the AUROC for the ability of the aGAPSS to predict IS was 0.686 [95% confidence interval (CI), 0.623–0.744; $P < 0.001$]. The ROC curve analysis showed that the cut-off aGAPSS for IS prediction was 10, with a sensitivity of 75.4% and a specificity of 60%. In the multivariable regression analysis, independent predictors of IS in the training cohort were age ($P < 0.001$), diabetes ($P = 0.030$), hyperuricemia ($P < 0.001$), the platelet count ($P = 0.001$), and aGAPSS > 10 ($P = 0.001$; Table 3). All VIF values were below 1.26, indicating low degrees of collinearity among variables. We built a predictive nomogram for IS (the aGAPSS-IS) based on these five independent predictors (Figure 2). For each patient, we added up the points identified on the points scale for the five risk factors. Then, the risk probability of IS was obtained according to the "Total Points" axis of the nomogram.

Validation of aGAPSS-IS score

For the training and validation cohorts, the AUROCs for the aGAPSS-IS were larger than those for the aGAPSS [0.853 (95% CI, 0.802–0.896) *vs.* 0.686 (95% CI, 0.623–0.744) and 0.793 (95% CI, 0.737–0.843) *vs.* 0.624 (95% CI, 0.560–0.656), respectively, both $P < 0.001$], meaning that the aGAPSS-IS showed better discriminative capacity (Figures 3A, B). The calibration plot for the training cohort showed optimal agreement between the aGAPSS-IS–predicted probability and the observed probability of IS; the mean absolute error was 0.015 (Figure 4A). The plot for the validation cohort also showed excellent concordance between these probabilities, with a mean absolute error of 0.028 (Figure 4B). For the training cohort, the DCA demonstrated that the aGAPSS-IS provided more net benefits than the aGAPSS for IS prediction when the threshold probability was >2% (Figure 5A). Similarly, the aGAPSS-IS always had marked net benefits over the aGAPSS for IS prediction when the threshold probability was >4% (Figure 5B).

Discussion

In the present study, we developed the clinical nomogram aGAPSS-IS for IS risk stratification for patients with APS. This nomogram was based on easily accessible data, including patient

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TABLE 1 Demographic and clinical variables of APS patients at baseline.

Variable	All cases (n = 478)	Training set (n = 239)	Validation set (n = 239)	P-value
Male, n (%)	112 (23.4)	61 (25.5)	51 (21.3)	0.28
Age (years), median (IQR)	41.0 (31.0-57.0)	42.0 (32.0-57.0)	41 (31-57)	0.94
BMI (kg/m^2), median (IQR)	23.6 (20.8-26.4)	23.7 (20.7-26.6)	23.6 (21.0-26.1)	0.71
Time from the first APS event (months), median (IQR)	11.0 (1.0-36.0)	8.0 (1.0-36.0)	8.0 (1.0-36.0)	0.71
aGAPSS, median (IQR)	10.0 (7.0-13.0)	11.0 (7.0-13.0)	10.0 (7.0-14.0)	0.99
Autoimmune disease, n (%)	216 (45.2)	115 (48.1)	101 (42.3)	0.20
Systemic lupus erythematosus, n (%)	152 (31.8)	80 (33.5)	72 (30.1)	0.432
Sjögren's syndrome, n (%)	34 (7.1)	15 (6.3)	19 (7.9)	0.477
rheumatoid arthritis, n (%)	19 (4.0)	12 (5.0)	7 (2.9)	0.242
systemic sclerosis, n (%)	11 (2.3)	6 (2.5)	5 (2.1)	0.760
Vascular thrombosis only, n (%)	299 (62.6)	156 (65.3)	143 (59.8)	0.22
Pregnancy morbidity only, n (%)	144 (30.1)	70 (29.3)	74 (30.9)	0.69
Vascular thrombosis and pregnancy morbidity, n (%)	35 (7.3)	19 (7.9)	16 (6.7)	0.60
Smoking, n (%)	58 (12.1)	29 (12.1)	29 (12.1)	1.00
Hypertension, n (%)	135 (28.2)	74 (31.0)	61 (25.5)	0.20
Hyperlipidemia, n (%)	248 (51.9)	130 (54.4)	118 (49.4)	0.27
Diabetes, n (%)	66 (13.8)	37 (15.5)	29 (12.1)	0.29
COPD, n (%)	6 (1.3)	4 (1.7)	2 (0.8)	0.69
Chronic kidney disease, n (%)	29 (6.1)	16 (6.7)	13 (5.4)	0.57
Hyperuricemia, n (%)	46 (9.6)	25 (10.5)	21 (8.8)	0.54
Anticoagulation, n (%)	184 (38.5)	95 (39.7)	89 (37.2)	0.57
Antiplatelet, n (%)	133 (27.8)	65 (27.2)	68 (28.5)	0.71
Immunosuppressant, n (%)	203 (42.5)	111 (46.4)	92 (38.5)	0.13
HCQ, n (%)	229 (47.9)	113 (47.3)	116 (48.5)	0.78
aCL, n (%)	299 (62.6)	149 (62.3)	150 (62.8)	0.93
aβ2GPI, n (%)	308 (64.4)	152 (63.6)	156 (65.3)	0.70
LAC, n (%)	281 (58.8)	142 (59.4)	139 (58.2)	0.78
Triple aPL positivity, n (%)	165 (34.5)	75 (31.4)	90 (37.6)	0.15
Platelet (×10^9/L), median (IQR)	151.0 (76.5-217.0)	153.0 (87.0-225.0)	155.0 (66.1-217.0)	0.31
Mean platelet volume (fl), median (IQR)	9.8 (8.6-10.9)	9.8 (8.6-10.9)	9.8 (8.6-10.9)	0.71
INR, median (IQR)	1.0 (0.9-1.1)	1.0 (0.9-1.2)	1.0 (0.9-1.2)	0.28
D-Dimer (ng/ml), median (IQR),	267.0 (100.0-580.0)	251.0 (94.0-564.0)	222.0 (92.0-544.0)	0.58
ESR increased, n (%)	203 (42.5)	98 (41.0)	105 (43.9)	0.52
CRP increased, n (%)	142 (29.7)	72 (30.1)	70 (29.3)	0.84
Low C3, n (%)	189 (39.5)	99 (41.4)	90 (37.7)	0.40
Low C4, n (%)	185 (38.7)	95 (39.7)	90 (37.7)	0.64
ANA positive, n (%)	279 (58.4)	146 (61.1)	133 (55.6)	0.23

IS, ischemic stroke; APS, antiphospholipid antibody syndrome; BMI, body mass index; IQR, interquartile range; aGAPSS, adjusted Global Anti-Phospholipid Syndrome Score; COPD, chronic obstructive pulmonary disease; HCQ, Hydroxychloroquine; LAC, lupus anticoagulant; aCL, anti-cardiolipin antibody; aβ2GPI, anti-β2-glycoprotein I antibody; aPL, antiphospholipid antibody; IQR, interquartile range; INR, international normalized ratio; C3, complement 3; C4, complement 4; ANA, antinuclear antibody.

age, diabetes, hyperuricemia, platelet count, and aGAPSS. It showed better performance than the aGAPSS for IS prediction in an APS cohort. It could facilitate rheumatologists in making individualized decisions about the clinical management of patients with APS (e.g., whether neuroimaging is indicated during hospitalization) by weighing the probability of IS occurrence.

IS represents the most common and disabling arterial involvement in APS (28). It is vital to identify APS patients at high risk of stroke and adopt timely prophylactic treatment measures. The aGAPSS, based on conventional cardiovascular risk factors and aPL profile, was a widely accepted risk stratification score (10, 16, 29). Consistent with this, our results demonstrated that aGAPSS > 10 could predict IS in APS patients with a sensitivity of 75.4% and a specificity of 60%. However, the performance of aGAPSS in discriminating IS was dissatisfactory, perhaps due to the lack of consideration of some critical risk factors.

TABLE 2 Univariate analysis of ischemic stroke occurrence based on the training cohort.

Variable	IS group (n = 69)	non-IS group (n = 170)	OR (95% CI)	P-value
Male, n (%)	25 (36.2)	36 (21.2)	2.115 (1.145-3.906)	**0.017**
Age (years), median (IQR)	54.0 (43.0-64.0)	37.0 (31.0-50.0)	1.055 (1.034-1.076)	**<0.001**
BMI (kg/m^2), median (IQR)	24.0 (21.3-27.3)	23.4 (20.7-26.4)	1.021 (0.958-1.087)	0.522
Time from the first APS event (months), median (IQR)	11.0 (2.0-48.0)	7 (1.0-33.2)	1.004 (1.000-1.009)	0.640
aGAPSS, median (IQR)	13.0 (11.0-16.0)	9.0 (5.3-13.0)	1.169 (1.088-1.256)	**<0.001**
Autoimmune disease, n (%)	40 (57.9)	75 (44.1)	1.97 (1.114-3.486)	**0.020**
Smoking, n (%)	12 (17.4)	17 (10.0)	1.895 (0.852-4.213)	0.117
Hypertension, n (%)	35 (50.7)	41 (24.1)	3.239 (1.798-5.834)	<0.001
Hyperlipidemia, n (%)	45 (65.2)	85 (50.0)	1.875 (1.050-3.347)	0.033
Diabetes, n (%)	21 (30.4)	12 (7.1)	8.464 (3.957-18.106)	**<0.001**
COPD, n (%)	3 (4.3)	1 (0.6)	7.682 (0.785-75.176)	0.080
Chronic kidney disease, n (%)	6 (8.7)	10 (5.9)	1.524 (0.531-4.369)	0.433
Hyperuricemia, n (%)	18 (26.1)	8 (4.7)	7.147 (2.934-17.409)	**<0.001**
Anticoagulation, n (%)	24 (34.8)	75 (44.1)	0.676 (0.378-1.207)	0.185
Antiplatelet, n (%)	24 (34.8)	42 (24.7)	1.625 (0.887-2.979)	0.116
Immunosuppressant, n (%)	36 (52.2)	75 (44.1)	1.273 (0.727-2.230)	0.398
HCQ, n (%)	30 (43.5)	85 (50.0)	0.769 (0.438-1.351)	0.361
aCL, n (%)	51 (73.9)	100 (58.8)	1.983 (1.069-3.680)	0.030
aβ2GPI, n (%)	46 (66.7)	57 (33.5)	1.009 (0.557-1.826)	0.977
LAC, n (%)	55 (79.7)	91 (53.5)	3.411 (1.763-6.596)	<0.001
Triple aPL positivity, n (%)	31 (44.9)	44 (25.9)	2.336 (1.301-4.195)	0.005
Platelet (×10^9/L), median (IQR)	114.9 (63.0-172.0)	180.1 (104.3-237.5)	0.993 (0.989-0.996)	**<0.001**
Mean platelet volume (fl), median (IQR)	9.5 (8.2-10.9)	9.9 (9.0-10.9)	0.932 (0.784-1.108)	0.423
INR, median (IQR)	1.0 (0.9-1.1)	1.0 (0.9-1.2)	0.912 (0.418-1.988)	0.817
D-Dimer (ng/ml), median (IQR),	178.0 (87.0-543.0)	279.0 (96.0-557.5)	1.000 (1.000-1.000)	0.207
ESR increased, n (%)	24 (34.8)	74 (43.5)	0.692 (0.387-1.237)	0.214
CRP increased, n (%)	22 (31.9)	50 (29.4)	1.123 (0.614-2.056)	0.706
Low C3, n (%)	35 (50.7)	64 (37.6)	1.705 (0.969-2.999)	0.064
Low C4, n (%)	33 (47.8)	62 (36.5)	1.597 (0.906-2.813)	0.105
ANA positive, n (%)	43 (62.3)	103 (60.6)	1.076 (0.605-1.914)	0.804

IS, ischemic stroke; BMI, body mass index; IQR, interquartile range; aGAPSS, adjusted Global Anti-Phospholipid Syndrome Score; COPD, chronic obstructive pulmonary disease; HCQ, Hydroxychloroquine; LAC, lupus anticoagulant; aCL, anti-cardiolipin antibody; aβ2GPI, anti-β2-glycoprotein I antibody; aPL, antiphospholipid antibody; INR, international normalized ratio; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; C3, complement 3; C4, complement 4; ANA, antinuclear antibody.
The provided bold values mean P-value < 0.05.

TABLE 3 Multivariate analysis of IS occurrence based on the training cohort.

Variables	β Coefficient	Multivariate analysis	
		OR (95% CI)	P-value
Age (years)	0.041	1.042 (1.018-1.066)	**<0.001**
Gender	0.661	1.937 (0.889-4.217)	0.096
Diabetes	1.033	2.810 (1.102-7.160)	**0.030**
aGAPSS (> 10)	1.281	3.601 (1.677-7.731)	**0.001**
Hyperuricemia	2.150	8.584 (2.758-26.723)	**< 0.001**
Platelet counts (×10^9/L)	-0.007	0.993 (0.988-0.997)	**0.001**
Autoimmune disease	0.322	1.380 (0.661-2.883)	0.391

IS, ischemic stroke; CI, confidence interval; OR, odds ratio; aGAPSS, adjusted Global Anti-Phospholipid Syndrome Score.
The provided bold values mean P-value < 0.05.

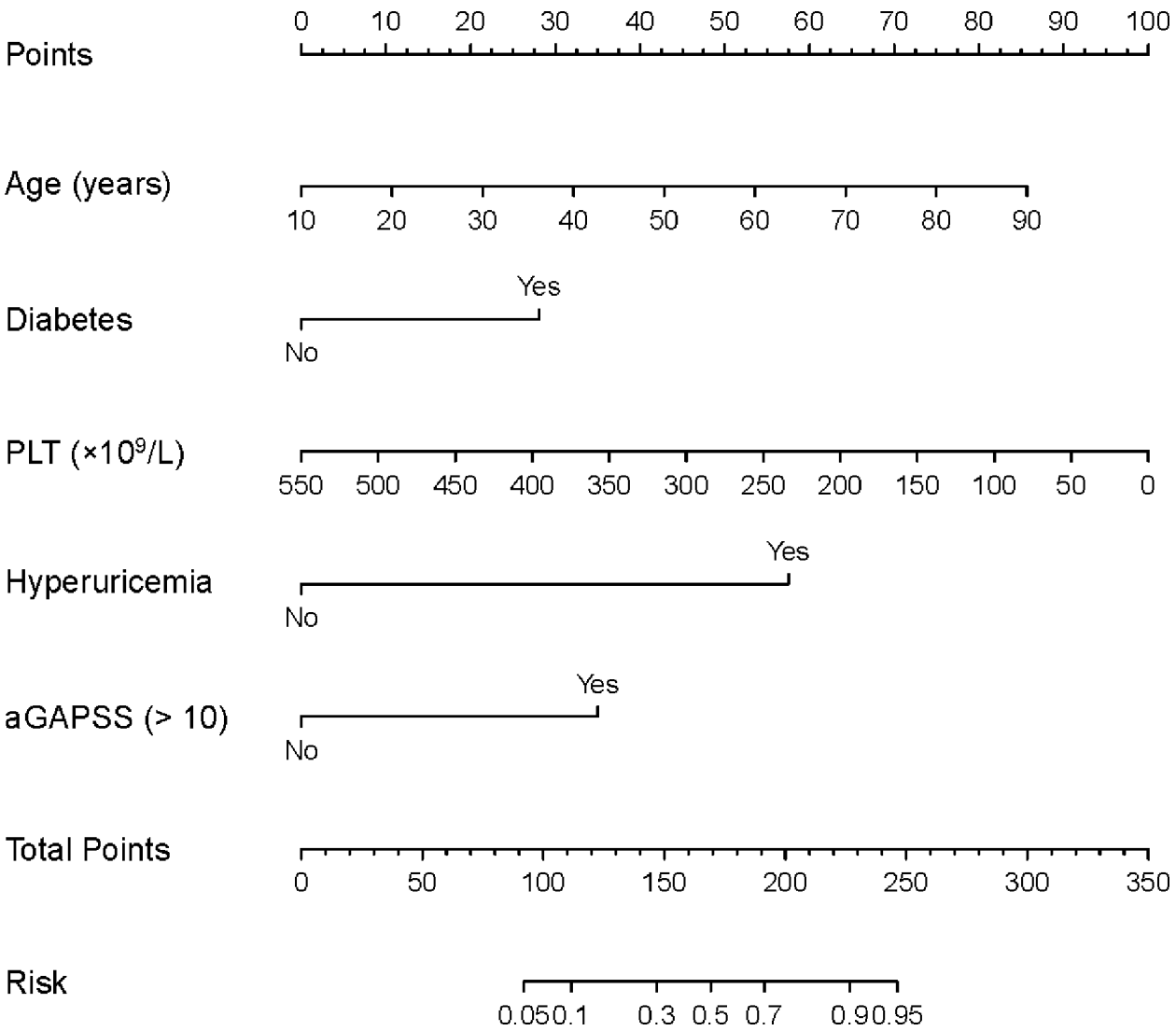

FIGURE 2
The nomogram for predicting the risk of IS in the training cohort. For each patient, we added up the points identified on the points scale for the five risk factors. Then, the risk probability of IS was obtained according to the "Total Points" axis of the nomogram. APS, antiphospholipid syndrome; IS, ischemic stroke; aGAPSS, adjusted Global Anti-Phospholipid Syndrome Score; PLT, platelet count.

Age is a robust non-modifiable risk factor for IS in the general population; the risk of IS doubles every 10 years after the age of 55, and almost 75% of strokes occur in people aged > 65 years (30). Patients with diabetes are more prone to atherosclerosis and microangiopathy than are healthy people, and these vascular diseases deteriorate rapidly, leading to cardiovascular accidents. The prevalence of diabetes among patients experiencing IS is estimated to be 33% and was found to be associated closely with poor outcomes and stroke recurrence in this population (31). Thus, aging and diabetes contribute to the incidence of IS in patients with APS. Hyperuricemia has a dose-response relationship to cardiovascular disease (23) and is an accepted risk factor for venous thromboembolism (32, 33). According to a longitudinal study including 15773 participants, a 59.5-μmol/l increase in uric acid was associated with a 28% increase in total and cardiovascular mortality during ten years of follow-up (34). Monosodium urate crystals can induce neutrophil extracellular trap release (35), a very important mechanism for thrombosis in APS (36). Hence, hyperuricemia is another independent risk factor for IS in patients with APS. Thrombocytopenia is related significantly to IS and is a risk factor for thrombosis in patients with APS (37) and aPL carriers (12). A prospective study including 228 APS patients demonstrated that patients with

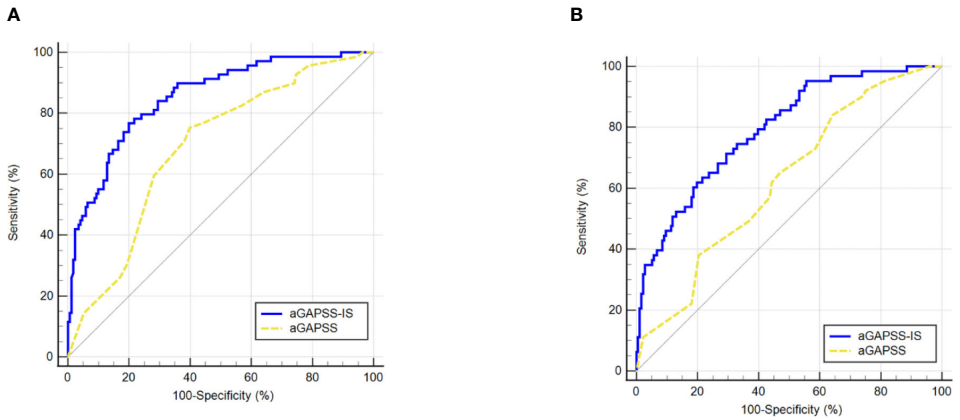

FIGURE 3
Comparison of the area under the receiver operating characteristic curve values between aGAPSS-IS score and aGAPSS in the training and the validation cohort. **(A)** In the training cohort, the aGAPSS-IS score had a larger AUROC than the aGAPSS [0.853 (95% CI, 0.802-0.896) *vs.* 0.686 (95% CI, 0.623-0.744), P < 0.001]; **(B)** In the validation cohort, the AUROC of aGAPSS-IS score was larger than the aGAPSS [0.793 (95% CI, 0.737-0.843) *vs.* 0.624 (95% CI, 0.560-0.656), P < 0.001]. aGAPSS, adjusted Global Anti-Phospholipid Syndrome Score.

thrombocytopenia had a higher risk of thrombotic events than those without [HR = 2.93, (95%CI:1.31-6.56)] (38). Phospholipids are integral parts of the platelet membrane, and the binding of aPL leads to the destruction of platelets and the release of microparticles, which play a procoagulant role in APS-related thrombotic events (39, 40). In our study, it was also an essential risk factor for arterial complications in the central nervous system.

The nomogram makes clinicians realize the critical role of traditional cardiovascular disease (CVD) risk factors for IS. CVD, especially stroke and coronary artery disease, is a leading cause of morbidity and mortality in APS (6). In the recent European League Against Rheumatism recommendations for managing CVD, the screening and strict control of traditional cardiovascular risk were highlighted in APS (41). However, CVD factors' (e.g., hypertension and dyslipidemia) target achievement was suboptimal in APS, especially in high/very high-risk patients (42). Therefore, modifiable risk factors for arterial events, such as hypertension, diabetes, and hyperuricemia, should be strictly monitored and controlled in patients with APS to reduce the risk of IS.

This study has several limitations. First, it was retrospective rather than prospective, which may have attenuated the significance of the findings. Second, to make the nomogram

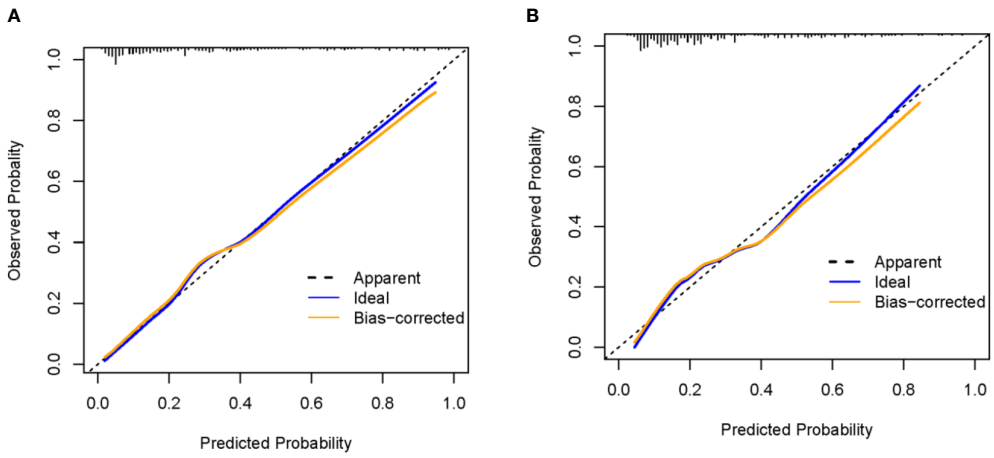

FIGURE 4
The calibration curve of the aGAPSS-IS score in the training and the validation cohort. **(A)** mean absolute error = 0.015 (training cohort); **(B)** mean absolute error = 0.028 (validation cohort).

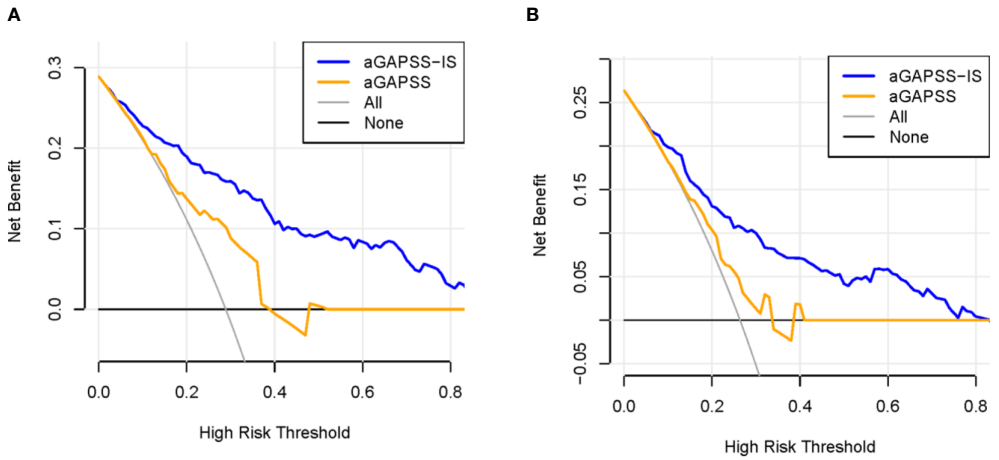

FIGURE 5
Comparison of the decision curves between the aGAPSS-IS score and aGAPSS in the training and the validation cohort. **(A)** For a threshold probability > 2%, application of the aGAPSS-IS score would add more net benefit to patients compared to the use of the aGAPSS in the training cohort. **(B)** For a threshold probability > 4%, the aGAPSS-IS score would provide more net benefit to APS patients than the aGAPSS in the validation cohort. aGAPSS adjusted Global Anti-Phospholipid Syndrome Score.

convenient in clinical practice, we did not incorporate valuable data from other antibodies (e.g., anti-phosphatidylserine/prothrombin), which may lead to some information loss. Thirdly, in our study, only 53.9% (184/334) of thrombotic APS were under long-term anticoagulation. Similarly, in a prospective study of 1000 APS patients, 40.2% received oral anticoagulants during the first 5 years and 37.0% during the second 5 years of the follow-up period (6). Low rates of anticoagulation prescription in patients at high risk of thrombosis (e.g., APS, atrial fibrillation combined with IS) may exist in different regions (6, 43–45), posing a challenge for thrombosis prevention. The possible reasons may be lower levels of education, lower income, and prior antiplatelet use (43). There is no sufficient evidence that anticoagulation reduces the risk of IS in our study. Thus, anticoagulation was not included in the nomogram. More prospective studies are expected to explore the association between anticoagulation and IS incidence. Finally, this study was based on clinical information from a single center's database and lacked external validation. Hence, we encourage the performance of multicenter studies to further validate the reliability and applicability of the aGAPSS-IS.

Conclusion

The aGAPSS-IS may be a good tool for the identification of patients with APS at high risk of IS based on routinely available data. It may aid physicians in making individualized treatment decisions for patients with APS by weighing the probability of IS occurrence.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by ethics committee at Peking University People's Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors were involved in the design of this study. CL and JZ conceived the original idea, supervised, and interpreted the result of this work. XS and YF performed the statistical analysis and wrote the manuscript. XS, YF, and JZ were involved in the assessment of ischemic stroke. YJ, GL, and ML contributed to clinical data collection pre-processing. YX gave advice in the statistical analysis and data interpretation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Thrombocytopenia in the first trimester predicts adverse pregnancy outcomes in obstetric antiphospholipid syndrome

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Background: Thrombocytopenia is a common manifestation of antiphospholipid syndrome (APS), and is a main concern for bleeding on the standard treatment of low dose aspirin (LDA) and low molecular weight heparin (LMWH) in obstetric APS (OAPS).

Objective: This study assesses the possible relationship between thrombocytopenia during the first trimester and adverse pregnancy outcomes (APOs) in OAPS patients.

Methods: A case-control study was conducted at Peking University People's Hospital, Beijing, China. The clinical, immunologic, and pregnancy outcomes of the OAPS patients were collected. Univariate and multivariate logistic regression analyses were applied to assess the relationship between APOs and thrombocytopenia in the first trimester.

Results: A total of 115 participants were included in the analysis. There were no difference on antepartum and postpartum hemorrhage between the two groups. The gestational age in the thrombocytopenia group was less than that in the control group (34.12 ± 8.44 vs. 37.44 ± 3.81 weeks, p = 0.002). Hypocomplementemia, double aPL positive, and high titers of anti-β2 glycoprotein I were more frequent in APS patients with thrombocytopenia (p < 0.05). Compared to the control group, thrombocytopenia in the first trimester was correlated with SGA (12.12% vs. 31.25%, p = 0.043), premature birth <37 weeks (16.16% vs 43.75%, p = 0.010) and intrauterine fetal death (2.02% vs 12.50%, p = 0.033). Thrombocytopenia in first-trimester independently increased the risk of preterm birth <37 weeks (OR = 5.40, 95% CI: 1.35-21.53, p = 0.02) after adjusting for demographic and laboratory factors. After adding medication adjustments, these factors above become insignificant (p > 0.05). Of note, the number of platelets increased after delivery in 14 thrombocytopenia patients with live fetuses (p = 0.03).

Conclusion: This study demonstrates that thrombocytopenia in the first trimester increases the risks of preterm birth in women with APS. The effective OAPS treatments may improve pregnancy outcomes and not increase the risk of antepartum and postpartum hemorrhage.

KEYWORDS

antiphospholipid syndrome, preterm birth, small-for-gestational age, thrombocytopenia, intrauterine fetal death

Introduction

Antiphospholipid syndrome (APS) is described as an autoimmune condition characterized by arterial or venous thrombosis and/or pregnancy complications, accompanied by persistent antiphospholipid antibodies (aPLs) (1, 2). The classical aPLs are anticardiolipin antibodies (aCL), anti-β2 glycoprotein I (aβ2GPI), and lupus anticoagulant (LA) (3, 4). Platelet activation is one stage of etiopathogenesis (4), while the binding of aPLs to β2GPI on platelets leads to its activation and aggregation (5).

Thrombocytopenia is a common hematologic manifestation in APS patients, with a prevalence ranging from 16 to 53% (6). In several studies, the frequencies of thrombocytopenia were higher than pregnancy morbidity (7, 8). Differences in prevalence partially depend on different threshold descriptions of thrombocytopenia ($<100\times10^9$/L or $<150\times10^9$/L). In a prospective study with individuals with platelet counts between 100 and 150×10^9/L, 88% of these platelet counts reached normal values or remained stable without any treatment during follow-up (9).

Several studies have investigated the relationship between thrombocytopenia and clinical APS features. Patients with thrombocytopenia had a higher incidence of cardiac valve thickening and dysfunction, epilepsy, chorea, arthritis, livedo reticularis, and skin ulcerations (10). However, another study did not find any correlations between thrombocytopenia and clinical manifestations in APS (11). The presence of thrombocytopenia could be a marker for high-risk APS patients (12).There have been few reports assessing the relationship between thrombocytopenia and pregnancy complications in APS patients.

The platelet counts decrease during normal pregnancy and increase postpartum (13). The timing of thrombocytopenia is also an important differentiate character from hypertension or gestational thrombocytopenia (14). Given the absence of management guidelines for immune thrombocytopenia in obstetric APS(OAPS), treatment recommendations for severe thrombocytopenia have typically been adapted from ITP (15). However, OAPS differ from ITP since these patients have hypercoagulability with thrombocytopenia. Low-dose aspirin (LDA) and low molecular weight heparin (LMWH) are currently accepted first-line treatments for OAPS (16), which are not included in ITP management. However, the treatment of LDA and LMWH also have the potential risk of thrombocytopenia. Therefore, physicians may avoid prescribing LDA and LMWH for fear of bleeding. Considering the high prevalence of low platelet and treatment dilemmas in OAPS, we sought to evaluate possible associations between thrombocytopenia and adverse pregnancy outcomes (APOs) in OAPS patients. What's more, thrombocytopenia throughout pregnancy cannot exclude gestational hypertension and physiological thrombocytopenia in the second and third trimester of pregnancy. The relationship between thrombocytopenia in the first trimester and APOs in APS patients is worth to explore.

Materials and methods

Study population

Data from all OAPS patients were retrospectively reviewed between January 2013 and June 2021 at Peking University People's Hospital. All patients fulfilled the Sydney classification criteria of APS (17). Thrombocytopenia was defined as platelet levels less than 100×10^9/L.

The inclusion criteria were: 1) live fetus diagnosed by ultrasonic examination at 6 weeks; 2) diagnosis of primary APS before or during pregnancy; 3) complete clinical and follow-up data. The exclusion criteria were: 1) voluntary interruption of pregnancy; and 2) thrombocytopenia caused by other causes, including hematological diseases, EDTA-dependent pseudo thrombocytopenia, and splenomegaly; 3) patients whose clinical data were missing, as well as those who were lost during follow-up.

This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of Peking University People's Hospital

(2019PHB252). All patients signed written informed consents before participating in this study.

Data collection and follow-up

Demographic and clinical data included age, gestational age, obstetric history, previous abortion history (early abortion<10 weeks, late abortion≥10 weeks), previous thrombosis, pregnancy, and fetal outcomes.

Laboratory data included antinuclear antibody (ANA), hypocomplementemia (low complement C3/C4), aCL, aβ2GPI, and LA. Treatment included prednisone (pred), hydroxychloroquine (HCQ), LMWH, LDA, intravenous immunoglobulin (IVIg), and calcineurin inhibitors.

Adverse pregnancy outcomes

Adverse pregnancy outcomes (APOs) were classified as follows: preeclampsia (18, 19); preterm birth <37 weeks gestational age excluding excessive membrane stretching, prior cervical surgery and choriodecidual infection (20); premature rupture of membranes (21); small-for-gestational-age (SGA) defined as birthweight below the 10th percentile (22–25); stillbirth defined as fetal death after 20 weeks (26); intrauterine fetal death defined as fetal death before 20 weeks (27).

Statistical analysis

Categorized variables are shown as a frequency or percentage, and continuous variables are shown as mean ± standard deviation (SD). The Mann-Whitney and Chi-square tests were used to determine any statistical difference between the means and proportions of the two groups. Both non-adjusted and multivariate-adjusted models were applied. Univariate and multivariate logistic regression analyses were applied to assess the relationship between APOs and thrombocytopenia. All analyses were performed with the statistical software packages R version 3.4.3 (http://www.R-project.org, The R Foundation) and EmpowerStats (http://www.empowerstats.com, X&Y Solutions, Inc., Boston, MA). A two-sided significance of 0.05 was considered statistically significant.

Results

Clinical characteristics of APS patients with or without thrombocytopenia in the first trimester

A total of 124 pregnant women were screened for in this study, and 9 participants were excluded because they did not meet the inclusion criteria. The remaining 115 patients were divided into the control group (243.52 ± 66.88×10^9/L, N = 99) and thrombocytopenia in the first-trimester group (54.06 ± 22.46×10^9/L, N = 16) according to the number of platelets. The platelet count of 6 participants was lower than 50×10^9/L (Table 1). Compared with the control group, the thrombocytopenia group had a lower previous abortion rate (77.78% vs. 37.50%, p = 0.001), especially lower early abortion rate (62.63% vs. 25.0%, p = 0.006). In the thrombocytopenia group, there was a lower rate of APS diagnosis before pregnancy (37.5% vs. 87.88%, p < 0.001).

The percentage of pregnancy by artificial insemination in the control group was higher than in the thrombocytopenia group (21.21% vs. 0.00%, p = 0.043). Gestational age in the thrombocytopenia group was less than that in the control group (34.12 ± 8.44 vs. 37.44 ± 3.81 weeks, p = 0.0023) (Table 1). Hypocomplementemia, single aPL positive, high titer of aβ2GPI and/or aCL, and LA positive were all more frequent in APS patients with thrombocytopenia in the first trimester (p<0.05) (Table 1). The prevalence of double aPL positive was higher in the thrombocytopenia group than in the control group (56.25% vs. 25.25%, p=0.011) (Table 1). There was no difference of antepartum (27.27% vs. 43.75%, p= 0.180) and postpartum (21.65% vs. 33.33%, p = 0.319) hemorrhage between the two groups.

The dosage and utilization rate of prednisone was higher in the thrombocytopenia group (75.00% of patients with a median dose of 13.75 mg/day) than in the control group (24.24% of patients with a median dose of 0.00 mg/day). The thrombocytopenia group was treated less frequently with LMWH (25.00% vs. 79.80%), aspirin (25.00% vs. 72.73%), and LMWH+aspirin (6.25% vs. 56.57%) than the control group (All p < 0.001). Additionally, therapeutic doses of LMWH and aspirin in the control group [4100.00 (4100.00-4100.00) IU/day and 75(0.00-100.00) mg/day, respectively] were significantly higher than in the thrombocytopenia group [0.00 (0.00-1025.00) IU/day and 0.00 (0.00-12.50) mg/day, respectively] (All p < 0.001). The use of IVIg in the thrombocytopenia group was higher than in the control group (25.00% vs. 2.02%, p < 0.001). (Table 1).

Relationship between thrombocytopenia in the first trimester and APOs

Among patients with thrombocytopenia in the first trimester, 31.25% patients (N=5) were combined with SGA, while the proportion was only 12% (N=12) in the control group (p = 0.043). Compared to the control group, thrombocytopenia in the first trimester was correlated with premature birth <37 weeks (16.16% vs 43.75%, p = 0.010) and intrauterine fetal death (2.02% vs 12.50%, p = 0.033) (Table 2). In the thrombocytopenia group, patients who were diagnosed with low platelet count

TABLE 1 Basic and gestational characteristics between the two groups.

	Thrombocytopenia group (N = 16)	Control group (N = 99)	*p*-value
Platelet counts ($\times10^9$/L)	54.06 ± 22.46	243.52 ± 66.88	<0.001*
Platelet counts ≤ 50×10^9/L, n (%)	6 (37.50)	0 (0.00)	<0.001*
Age, years	31.5 ± 4.1	32.8 ± 3.8	0.289
Age ≥ 35 years, n (%)	2 (12.50)	20(20.20)	0.467
Previous abortion, n (%)	6 (37.50)	77 (77.78)	0.001*
Early abortion, n (%)	4 (25.00)	62 (62.63)	0.006*
Late abortion, n (%)	2 (12.50)	32 (32.32)	0.112
History of thrombosis, n (%)	0 (0.00)	5 (5.05)	0.361
Nulliparity, n (%)	14 (87.50)	71 (71.72)	0.182
Diagnosis of APS before pregnancy, n (%)	6 (37.50)	87 (87.88)	<0.001*
Gestational age, weeks	34.12 ± 8.44	37.44 ± 3.81	0.023*
Full term birth, n (%)	9 (56.25)	85 (85.86)	0.006*
Pregnancy by artificial insemination, n(%)*	0 (0.00)	21 (21.21)	0.043*
Live fetus, n (%)	14 (87.50)	96(96.97)	0.142
antepartum hemorrhage, n (%)	7 (43.75)	27 (27.27)	0.180
Postpartum hemorrhage, n (%)	5 (33.33)	21 (21.65)	0.319
Amount of bleeding(ml)	436.67 ± 316.49	320.10 ± 244.91	0.176
ANA positive, n (%)	5 (35.71)	21 (25.61)	0.432
ANA titer, n (%)			0.525
1:40	1 (7.14)	11(13.41)	
1:80	2 (14.29)	4 (4.88)	
1:160	1 (7.14)	4 (4.88)	
1:320	1 (7.14)	2 (2.44)	
Hypocomplementemia, n (%)	6 (37.50)	16 (16.16)	0.042*
High titer of aβ2GPI and/or aCL, n (%)	11 (68.75)	27 (27.27)	<0.001*
LA positive, n (%)	9 (56.25)	22 (22.22)	0.004*
Double aPL positive, n (%)	9 (56.25)	25 (25.25)	0.011*
Triple aPL positive, n (%)	4 (25.00)	9 (9.09)	0.060
Anti-dsDNA titer, IU/ml	7.69 ± 7.06	6.85 ± 5.87	0.808
Anti-dsDNA positive, n (%)	0 (0.00%)	1 (1.01%)	0.999
Without Treatment, n (%)	1 (6.25)	2 (2.02)	0.320
Pred, mg/d	13.75 (7.50-16.25)	0.00 (0.00-0.00)	<0.001*
Pred, n (%)	12 (75.00)	24 (24.24)	<0.001*
HCQ, mg/d	400.00 (200.00-400.00)	400.00 (0.00-400.00)	0.470
HCQ, n (%)	13 (81.25)	68 (68.69)	0.284
LMWH, IU/d	0.00 (0.00-1025.00)	4100.00 (4100.00-4100.00)	<0.001*
LMWH, n (%)	4 (25.00)	79 (79.80)	<0.001*
Aspirin, mg/d	0.00(0.00-12.50)	75(0.00-100.00)	<0.001*
Aspirin, n (%)	4 (25.00)	72 (72.73)	<0.001*
Aspirin+ LMWH, n (%)	1 (6.25)	56 (56.57)	<0.001*
IVIg, n (%)	4 (25.00)	2 (2.02)	<0.001*

*P < 0.05. APS, antiphospholipid syndrome; ANA, antinuclear antibody (ANA); hypocomplementemia, low C3/C4: complement C3/C4; aPLs: antiphospholipid antibodies included cardiolipin antibodies (aCL), anti-β2 glycoprotein I (aβ2GPI), and lupus anticoagulant (LA); Pred, prednisone; HCQ, hydroxychloroquine; LMWH: low molecular weight heparin, IVIg, Intravenous Immunoglobulin Therapy.

during pregnancy have a higher rate of intrauterine fetal death compared to the patients who were known to have low platelet count (100% vs 0.00%, $p = 0.008$) (Supplementary Table 1).

Univariate logistic regression analysis (Table 3) indicated that thrombocytopenia in the first trimester was associated with premature birth < 37 weeks (OR = 4.08, 95%CI: 1.33-12.55, $p = 0.01$). Thrombocytopenia in the first trimester had an increased risk of APOs, including SGA (OR=3.33, 95%CI: 0.99-11.26, $p = 0.05$) and intrauterine fetal death (OR=7.00, 95%CI: 0.91-53.75, $p = 0.06$).

TABLE 2 Adverse pregnancy outcomes in the thrombocytopenia in the first-trimester group and control group.

	Thrombocytopenia group (N = 16)	Control group (N = 99)	*p*-value
SGA, n (%)	5 (31.25)	12 (12.12)	0.043*
Preeclampsia n (%)	1 (6.25)	9 (9.19)	0.716
Premature birth <37 weeks, n (%)	7 (43.75)	16 (16.16)	0.010*
Stillbirth, n (%)	0 (0.00)	2 (2.02)	0.568
Intrauterine fetal death, n (%)	2 (12.50)	2 (2.02)	0.033*
PROM, n (%)	1 (6.25)	25 (25.25)	0.095

*$P < 0.05$. SGA, small for gestational age; PROM, Premature rupture of membranes.

The multivariate analysis of the relationship between thrombocytopenia in the first trimester and APOs are presented in Table 3. Thrombocytopenia in the first trimester independently increased the risk of premature birth <37 weeks (Model I: OR = 5.22, 95% CI: 1.43-19.07, $p = 0.01$; Model II: OR = 5.40, 95% CI: 1.35-21.53, $p = 0.02$). In the adjusted models I and II, the relationship between thrombocytopenia in the first trimester and SGA were not significant ($P > 0.05$) compared with the control group.

After adding the adjustments of therapeutic medication such as prednisone, HCQ, IVIg, LDA, and LMWH, with single or combined usage, the influences on APOs mentioned above become insignificant, which indicates that treatment could decrease the risk of APOs with thrombocytopenia ($p > 0.05$ as shown in Table 4).

TABLE 3 Univariate and multivariate logistic regression analyses of thrombocytopenia in first-trimester associated with adverse pregnancy outcomes in patients with APS.

Thrombocytopenia in first-trimester	Unadjusted	Model I	Model II
SGA	3.33 (0.99, 11.26) $p = 0.05$	2.48 (0.64, 9.58) $p = 0.18$	3.68 (0.81, 16.77) $p = 0.09$
Premature birth <37 weeks	4.08 (1.33, 12.55) $p = 0.01$	5.22 (1.43, 19.07) $p = 0.01$	5.40 (1.35, 21.53) $p = 0.02$
Intrauterine fetal death	7.00 (0.91, 53.75) $p = 0.06$	NA	NA

SGA, small for gestational age.
Adjust I model adjust for: age; previous abortion history; pregnancy by artificial insemination
Adjust II model adjust for: age; previous abortion history; pregnancy by artificial insemination. hypocomplementemia; high titer of aβ2GPI and/or aCL.
NA, Not Applicable.

Changes in platelets at different trimesters of pregnancy in thrombocytopenia patients

The following analysis compared the number of platelets in different pregnancy trimesters of 14 thrombocytopenia patients with a live fetus, including the first trimester, the second trimester, the third trimester, and 42 days postpartum (Supplementary Figure 1). The results indicate that the number of platelets increased after delivery with the treatment (57.64 ± 5.77 vs. $86.79 \pm 9.65 \times 10^9$/L, $p = 0.03$).

Discussion

Our study found that the prevalence of thrombocytopenia in the first trimester was 13.8% in OAPS patients. The rate of thrombocytopenia in our study was slightly lower than in a previous study (6), which could be due to differences in our defined platelet threshold and thrombocytopenia we counted in the first trimester not the whole pregnancy.

Several studies reported that the mechanism of thrombocytopenia in APS could be platelet consumption and/or destruction mediated by aPLs (28, 29). Platelets could also play a central role in APS as the main target of β2GPI (30, 31). Therefore, thrombocytopenia could be a predictive factor of clinical manifestations, including obstetrical morbidity in APS (32–34). In our study, hypocomplementemia and single/double-positive aPLs were found to be more frequent in APS patients with thrombocytopenia in the first trimester, which could indicate the pathogenic effect of antiphospholipid antibodies on the platelets.

Many studies reported that previous thrombosis, triple aPLs positivity, and LA positive were the factors related to various APOs (35–37). Little research reported the association between thrombocytopenia in the first trimester and pregnancy outcomes. Our study demonstrated that thrombocytopenia in the first trimester was correlated with SGA, premature birth <37 weeks and intrauterine fetal death compared to the control group. What's more, thrombocytopenia in the first trimester independently increased the risk of premature birth <37 weeks after adjustment for demographic and immunologic confounders. A recent study showed that rates of intrauterine growth retardation, recurrent fetal loss, and pregnancy toxemia were similar in APS patients with and without thrombocytopenia (10). These results differ from previous studies, which could be due to the study population and the definition of APOs. More importantly, the factors causing this difference may be thrombocytopenia in previous papers cannot completely exclude pregnancy induced hypertension or thrombocytopenia caused by gestational thrombocytopenia in the second and third trimester. Thrombocytopenia during the

TABLE 4 Medication treatment changed the adverse pregnancy outcomes in APS patients with thrombocytopenia in first-trimester.

Thrombocytopenia in first-trimester	Model I	Model II	Model III
SGA	3.70 (0.76, 18.05) $p = 0.11$	1.84 (0.32, 10.62) $p = 0.49$	0.67 (0.07, 6.64) $p = 0.73$
Premature birth <37 weeks	4.75 (1.13, 20.03) $p = 0.03$	3.17 (0.66, 15.28) $p = 0.15$	2.13 (0.34, 13.34) $p = 0.42$
Intrauterine fetal death	NA	NA	NA

SGA, small for gestational age.
Adjust I model adjust for: age; previous abortion history; pregnancy by artificial insemination; hypocomplementemia; High titer of 2gp1 and/or ACL; aspirin+low molecular weight heparin.
Adjust II model adjust for: age; previous abortion history; pregnancy by artificial insemination; hypocomplementemia; high titer of aβ2GPI and/or aCL; pred; IVIg.
Adjust III model adjust for: age; previous abortion history; pregnancy by artificial insemination; hypocomplementemia; high titer of aβ2GPI and/or aCL; pred; HCQ; aspirin; low molecular weight heparin; aspirin+low molecular weight heparin; IVIg.
NA, Not Applicable.

first trimester, which is usually the first time many patients receive a blood test, is an indicator for APOs in APS patients.

Our results showed the therapeutic dose of LMWH and aspirin in the thrombocytopenia group is lower than the dose in the control group, which is in line with the guidelines. Aspirin and LMWH did not increase the risk of poor pregnancy outcomes as previously reported (5). Additionally, in our study, anti-coagulation therapy did not increase the incidence of antepartum bleeding in patients with thrombocytopenia. The data showed that after adjusting the treatment factors, the prognosis of APS patients with thrombocytopenia could be improved. It is recommended that full-dose enoxaparin be provided for patients with platelet counts greater than 50×10^9/L and half-dose enoxaparin for platelet counts between 25×10^9/L and 50×10^9/L (38). These findings indicate that pregnant APS patients with thrombocytopenia in the first trimester should receive positive treatment.

Our findings showed that the dosage and utilization rate of prednisone was much higher in the thrombocytopenia group. Glucocorticoids are the first-line treatment for APS-associated immune thrombocytopenia (ITP) (15, 39). For pregnant women with ITP, the optimal dose of corticosteroids has not been determined. It is reported that treatment with corticosteroids at doses ≥15 mg/day during pregnancy and delivery is correlated with a higher incidence of complications in ITP mothers and infants, including premature labor and preeclampsia in the mother and abnormal body weight and congenital abnormalities in the infant (40). Low-dose corticosteroids (≤10 mg/day) could produce better results, even though the platelet counts are lower during the pregnancy period (40). Another study found that GC therapy of 1 mg/kg for ITP patients during pregnancy is less efficient than the non-pregnant population and increases the incidence of hypertensive disorders (41). A recent study showed that low doses of prednisone (5 mg once a day) and aspirin (75 mg once a day) were associated with a significant reduction in the titer of aβ2GPI, and women who successfully delivered exhibited significantly greater reductions of aβ2GPI than patients who experienced a fetal loss (42). In our study, the median daily dose of prednisone is 13.75 mg, which does not increase the risk of adverse pregnancy outcomes due to thrombocytopenia. These findings could suggest that lower starting doses of prednisone are appropriate in pregnant women with thrombocytopenia.

Our study has several limitations. First, it is a retrospective design, and we do not have complete information on all APS patients. Second, thrombocytopenia fluctuated in most patients, but we tried our best to record a similar time in the first trimester. Third, the sample size is relatively small, and therefore, we could not perform a stratified analysis. As such, we recommend conducting prospective cohort studies in a larger population.

Conclusion

The study shows that thrombocytopenia in the first trimester increases the risk of premature birth at <37 weeks in OAPS patients. Effective treatments may improve pregnancy outcomes without increasing the risk of antepartum and postpartum hemorrhage. These results are important for improving the treatment of APS patients with thrombocytopenia.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Peking University People's Hospital (2019PHB252). The patients/participants provided their written informed consent to participate in this study.

Author contributions

JJ, XX, JL, ML, and CL conceived of and designed the project. LH and YH collected and input the clinical and laboratory data. JJ completed the statistical analyses. JJ, ML, and CL conducted the table and figure calculations. JJ and CL wrote the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript. All authors discussed the results and contributed to the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.971005/full#supplementary-material

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Comparison of non-criteria antiphospholipid syndrome with definite antiphospholipid syndrome: A systematic review

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Objectives: Patients with laboratory or clinical manifestations suggestive of antiphospholipid syndrome (APS) but not fulfilling the classification criteria constitute a clinical challenge. This study aims to compare non-criteria APS (NC-APS) with definite APS in terms of clinical manifestations, therapies, and outcomes.

Methods: A systematic review of observational studies comparing definite and NC-APS was performed searching four electronic databases. Data on clinical manifestations, therapies and clinical outcomes was extracted.

Results: Sixteen studies, assessing a total of 3,798 participants, were included. Seven out of 10 studies found no significant difference in the prevalence of arterial or venous thrombosis between definite and NC-APS, with two studies on seronegative APS also finding no difference in thrombosis recurrence. Seven out of 12 studies found no significant difference in the prevalence of obstetric manifestations between groups, with the remaining exhibiting conflicting results. In 9 studies comparing treatment frequency in obstetric patients, all but one described similar treatment frequency, with the percentage of NC-APS treated during pregnancy ranging from 26% to 100%. In 10 studies comparing pregnancy outcomes of NC-APS versus definite APS, 7 found similar successful pregnancies/live births. Additionally, 5 studies described improvement of live

births in both groups with treatment, with three signalling aspirin monotherapy as efficacious as combination therapy in NC-APS.

Conclusion: This review hints at an absence of marked differences in most evaluated parameters between definite and NC-APS, emphasizing the value of a more active follow-up of these patients. The low-quality available evidence highlights the need for well-defined NC-APS populations in future studies.

Systematic Review Registration: https://www.crd.york.ac.uk/prospero, identifier CRD42020210674.

KEYWORDS

antiphospholipid syndrome, treatment, clinical manifestations, non-criteria, seronegative, probable, low titre, antiphospholipid antibodies

Introduction

Patients with laboratory or clinical manifestations suggestive of antiphospholipid syndrome (APS) but not fulfilling the Sydney Classification Criteria for definite APS (1) constitute a relevant challenge in clinical practice. Since the description of seronegative APS (SN-APS) by Hughes and Khamashta in 2003 (2), various publications have discussed the existence and characteristics of these patients (3–6). Furthermore, other elements not included in the classification criteria drew attention, namely "non-criteria" clinical manifestations (1, 5, 7–9). The report of the *14th International Congress on Antiphospholipid Antibodies Technical Task Force on APS clinical manifestations* highlighted the role of some of these features on the clinical course of the disease (5). Accordingly, efforts for the development of new classification criteria are currently underway, with the prospect of the eventual inclusion of some of these manifestations (10).

From a laboratory perspective, a growing number of publications have suggested that a larger number of patients would be classified as APS if the array of antibodies tested was expanded to include non-criteria antiphospholipid antibodies (aPL) (3, 11–16). Finally, even the current laboratory criteria raise questions, namely in the matter of the relevance of low titres of anticardiolipin (aCL) and anti-β2 glycoprotein I (anti-β2GPI) antibodies (17–21).

Consequently, there is a substantial number of patients who fit the profile of "non-criteria" APS (NC-APS) and a rising number of studies that include these patients; however, the small samples and the different definitions of non-criteria patients greatly undermine the formulation of generalizable conclusions (5, 22, 23). We recently elaborated a nomenclature proposal for research purposes (24) and categorized patients who do not fulfil the classification criteria in four subsets, under the broad term NC-APS. A review of the available data can enable a deeper understanding of their clinical characteristics and prognosis in comparison with patients with definite APS. Additionally, analysing potential subsets of "non-criteria" APS can help clarify discrepancies and similarities among them and suggest possible management specificities.

Therefore, we performed a systematic review of studies comparing patients with NC-APS with patients with definite APS in terms of the frequency of clinical manifestations (vascular thrombosis and pregnancy morbidity), prescribed therapies and reported clinical outcomes.

Methods

The systematic review protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO) on October 24, 2020, with registration number CRD42020210674.

Eligibility criteria

We included retrospective cohort studies comparing participants with NC-APS with those with definite APS regarding their clinical manifestations, therapies, and outcomes. The term NC-APS was considered to include patients with clinical and/or laboratory manifestations suggestive of APS but not fulfilling the Sydney Classification Criteria for Definite APS (1). Case reports and papers focusing exclusively on paediatric populations were excluded, as the disease is understudied and carries certain specificities in this age group (e.g., the absence of obstetric morbidity/pregnancy in most patients). No language or geographical restriction was applied. Conference abstracts were not excluded.

Search strategy

A literature search was conducted on the following bibliographic databases: CENTRAL, EMBASE, PubMed, and Web of Science. The search strategies were drafted by two authors and refined by an experienced librarian. The final search strategies for each database are presented in Supplementary Table 1. The search in each database was performed from inception. A first literature search was performed on August 20, 2020, followed by an update on January 30, 2022. In addition, we screened the references of retrieved articles for potentially relevant publications.

Study selection and data extraction

After duplicates removal, two reviewers independently evaluated the titles and abstracts of retrieved publications and, subsequently, the full text of selected articles. Disagreements on study selection were resolved by consensus.

Two reviewers independently extracted data from each of the included primary studies using a prespecified form. Differences were settled through an assessment conducted by a third reviewer. Authors of individual studies were contacted for clarification when needed. Data was extracted on the following: article characteristics (authors, year of publication, country of origin), study aims/purpose, study population, methodology, number of participants with "non-criteria" and definite APS, reported clinical manifestations, treatments, and outcomes. Clinical manifestations were grouped into vascular thrombosis (i.e., arterial, venous, or both) and obstetric morbidity (i.e., more than three abortions before 10 weeks of pregnancy, abortion after 10 weeks of pregnancy, and premature birth before 34 weeks of pregnancy). Information on therapies consisted of the use of low-dose aspirin and anticoagulation. Assessed outcomes included thrombosis recurrence and pregnancy outcomes (i.e., foetal loss or live birth) with or without treatment.

In addition to the global comparative analysis between NC-APS and definite APS patients, whenever such data were available, participants were also classified according to the following subsets of "non-criteria" APS (in the subgroup they more closely fitted) based on the nomenclature we previously proposed (24):

- "Seronegative APS": patients with clinical manifestations fulfilling APS classification criteria, plus the presence of "non-criteria" manifestations, persistently negative aPL, and exclusion of other thrombophilias that justify their whole clinical presentation. Although not included in our proposal, patients without non-criteria manifestations were also included in this subgroup since they are classified as SN-APS in various studies.
- "Clinical non-criteria APS" (CNC-APS): patients with "non-criteria" manifestations, plus aPL positivity fulfilling the APS classification criteria.
- "Incomplete laboratory APS": patients with clinical manifestations fulfilling APS classification criteria, plus two or more determinations of aCL between the 95th and 99th percentiles (or positive aCL determinations according to the commercial kit used but below 40 GPL or MPL), and/or two or more determinations of anti-β2GPI antibodies between the 95th and 99th percentiles (low titre patients).
- "Laboratory non-criteria APS" (LNC-APS): patients with clinical manifestations fulfilling APS classification criteria, negative or low titre classification criteria aPL, and positive "non-criteria" aPL testing.
- "Single-positive APS" (SP-APS): Although excluded from our nomenclature proposal, we included this additional group of patients frequently present in the literature to allow for the appraisal of the largest amount of available evidence. This subset includes patients with clinical manifestations fulfilling APS classification criteria and only one single positive determination of criteria aPL.

Quality assessment

The studies included in the systematic review were evaluated for their risk of bias by using the Newcastle-Ottawa scale (NOS) (25), which, for cohort studies, consists of three parameters (with a total of 8 subitems) - selection of study groups, comparability of groups, and the ascertainment of outcome. The maximum score for these three subsets is 9. Two reviewers independently graded the studies, and any differences were settled through an assessment done by a third reviewer.

Results

Search results

Initial database search yielded 6,995 references. After duplicates removal, 5,070 citations were identified. Based on their titles and abstracts, 5,023 records were excluded, and 47 full-text articles were retrieved and assessed for eligibility. Of these, 31 were excluded for the following reasons: 16 assessed patients who did not meet the eligibility criteria, eight presented data already described in other publications, while seven described "non-criteria" APS patients but did not compare them with patients with definite APS. The remaining 16 studies were included in the review (Figure 1) (4, 12, 26–39), of which one was a conference abstract (36) and the remainder

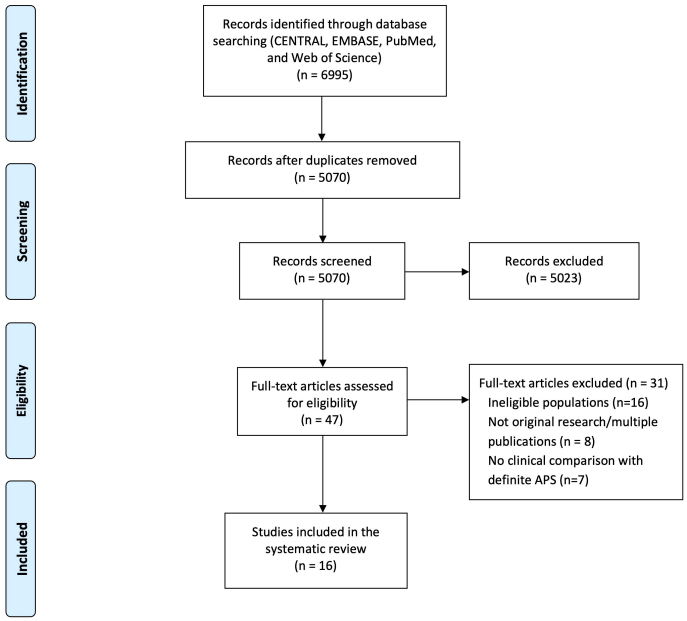

FIGURE 1
PRISMA flow diagram for the systematic review, detailing database searches, number of abstracts screened, and full texts retrieved.

full papers. In ten of the included articles, the specific comparison of outcomes between non-criteria and definite APS was the main objective (4, 26, 30–34, 36–38).

Description of included studies

The included studies were published between 2012 and 2021 and assessed a total of 3,798 participants (range 36-1,640 per study) (4, 12, 26–39), with 96.7% of female patients. Two studies derive from multicentric European registries (26, 38), and the remainder were performed in eight different countries. Most studies originated from Western Europe (4, 12, 26–29, 32, 33, 38), followed by Asia (30, 31, 37, 39), North Africa (35), the Middle East (34) and South America (36). Consecutive sampling was used in most studies (n=11) (4, 12, 27–30, 32–35, 37), while one study used a convenience sample (26), and three did not specifically state their sampling method (31, 36, 39). Thirteen studies provided information on the comparison between the clinical manifestations of non-criteria and definite APS (4, 12, 26–28, 30, 32–36, 38, 39), while nine studies compared outcomes and therapies used (4, 26, 29–33, 37, 38). A summarized description of included studies is presented in Table 1, and a more detailed description of each study´s aims and population are provided in Supplementary Table 2. Regarding quality assessment, the mean NOS score value for the 16 included studies was 6.8, with the detailed results available in Table 2.

Comparison between the clinical manifestations of non-criteria and definite APS

Vascular thrombosis

Twelve studies reported on the prevalence of vascular thrombosis in non-criteria APS versus definite APS. Two limited to the description of the prevalence in each group (12, 28), while 10 performed a statistical comparison between groups. The vast majority (7 studies) found no significant difference in the prevalence of thrombosis between NC-APS versus definite APS (1,088 participants) (4, 30, 33–36, 39). Three studies reported vascular events as more common in definite APS (596 participants) (27, 37, 38), but in one case the definite APS group comprised only triple-positive individuals (27) and in another study the authors discuss a possible selection bias due to the employed inclusion criteria (38). No studies reported thrombosis as more frequent in NC-APS. These findings were globally maintained when evaluating specifically venous or arterial thrombosis, and concerned specially the seronegative (5 studies), laboratory

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TABLE 1 Description of studies included in the systematic review by country, methodology and number of patients in each group.

Author, year (reference)	Country of origin	Methodology	Number of Patients						
			Definite APS	NC-APS (global)	SN-APS	CNC-APS	Incomplete APS	LNC-APS	Single positive APS
Mekinian, 2012 (32)	France	Retrospective	25	53	21	–	32	–	–
Rodriguez-Garcia, 2012 (4)	United Kingdom	Retrospective	87	67	67	–	–	–	–
Conti, 2014 (12)	Italy	Retrospective	25	24	24	–	–	–	–
Ofer-Shiber, 2015 (34)	Israel	Retrospective	126	117	–	–	117	–	–
Mekinian, 2016 (33)	France	Prospective	83	96	31	–	–	65	–
Omar, 2018 (35)	Egypt	Retrospective	30	30	30	–	–	–	–
Signorelli, 2017 (36)	Brazil	Retrospective	77	13	–	–	–	–	13
Fredi, 2018 (29)	Italy/France (3 centers)	Retrospective	85	81	–	81	–	–	–
Litvinova, 2018 (28)	France	Prospective	41	17	17	–	–	–	–
Shi, 2018 (39)	China	Retrospective	186	48	48	–	–	–	–
Alijotas-Reig, 2019 (26)	30 centers in 10 European countries	Retrospective and Prospective	1000	640	–	289	175[1]	–	175[1]
Abisror, 2020 (38)	14 centers in 5 European countries	Retrospective	285	187	–	–	–	187	–
Ferreira, 2020 (27)	France (3 centers)	Prospective	15[1]	21	21	–	–	–	–
Li, 2020 (30)	China	Prospective	34	94	–	94[1]	94[1]	–	94[1]
Lo, 2020 (31)	Taiwan	Retrospective	12	24	–	–	–	17	7
Yang, 2021 (37)	China	Retrospective	56	32	–	–	–	–	–

[1]NC-APS group includes mixed patients from various subsets
APS, Antiphospholipid Syndrome; CNC-APS, Clinical non-criteria APS; LNC-APS, Laboratory non-criteria APS; NC-APS, Non-criteria antiphospholipid syndrome; SN-APS, Seronegative Antiphospholipid Syndrome.
[1]Only triple-positive APS patients.

TABLE 2 Quality assessment of included studies by the Newcastle-Ottawa Scale.

Author, year (reference)	Selection (0 to 4)	Comparability (0 to 2)	Outcome (0 to 3)	Total (0 to 9)
Abisror, 2020 (38)	3	0	3	6
Alijotas-Reig, 2019 (26)	4	0	3	7
Conti, 2014 (12)	4	0	3	7
Ferreira, 2020 (27)	3	0	3	6
Fredi, 2018 (29)	4	0	3	7
Litvinova, 2018 (28)	4	0	3	7
Li, 2020 (30)	4	0	3	7
Lo, 2020 (31)	3	0	3	6
Mekinian, 2012 (32)	4	0	3	7
Mekinian, 2016 (33)	4	0	3	7
Ofer-Shiber, 2015 (34)	4	0	3	7
Omar, 2018 (35)	4	1	3	8
Rodriguez-Garcia, 2012 (4)	4	1	3	8
Signorelli, 2017 (36)	2	0	3	5
Shi, 2018 (39)	3	0	3	6
Yang, 2021 (37)	4	0	3	7

non-criteria (3 studies) and incomplete laboratory (2 studies) subgroups of NC-APS. Regarding thrombosis recurrence, two studies analysing SN-APS patients found no significant difference in comparison to definite APS, even though the duration of follow-up was not reported (4, 35). A summary of these results is available in Table 3.

Obstetric morbidity

Fourteen studies compared the prevalence of obstetric morbidity in NC-APS versus definite APS. Two studies solely described the prevalence in each group (12, 28), while 12 performed a statistical comparison. The majority of these studies (seven) found no statistical differences in the global prevalence of pregnancy morbidity (970 participants) (4, 29, 32–36), 3 studies found obstetric manifestations as more common in definite APS than in NC-APS (1,962 participants) (26, 37, 39), while the opposite occurred in 2 studies (508 participants) (27, 38). These results were globally maintained when evaluating specific Sydney criteria manifestations (i.e., three or more spontaneous abortions before 10 weeks of gestation, fetal death, and prematurity before 34 weeks of gestation). In a large European registry on obstetric APS (EUROAPS) (26), a noticeable difference between these groups was the chronology of placental vasculopathy – predominantly prior to 34 weeks in definite APS and subsequent to 34 weeks in NC-APS patients. These studies evaluating obstetric morbidity encompassed the seronegative (6 studies), incomplete laboratory (3 studies), laboratory non-criteria, clinical non-criteria, and single-positive (2 studies each) subgroups of NC-APS. A summary of these results is available in Table 3.

Comparison between the treatment and outcomes of "non-criteria" and definite APS

Treatment frequency and regimens

Only one study compared the treatment frequency of thrombotic NC-APS (specifically SN-APS) and definite APS patients, with no significant difference in the percentage of patients under anticoagulation between groups (59.6% for SN-APS versus 60.8% for definite APS) (4).

Concerning obstetric APS, 9 studies reported a comparison of the treatment frequency and regimens in non-criteria versus definite APS, with all but one describing similar treatment frequency (2,762 participants) (4, 26, 29–32, 37, 38), and one reporting more frequent treatment in definite obstetric APS (179 participants) (33). Of note, the aspirin dosage was not discriminated in 5 studies (4, 31, 37, 38), it was referred as "low-dose aspirin" in two studies (26, 29), and specified as 100 mg/day in one study (32) and 50-75 mg/day in another (30). In two of the studies the use of aspirin/LMWH was more common in definite APS (29, 33), and in another the study this was the case for the use of therapeutic dose of LMWH (38). The reported percentage of NC-APS patients submitted to treatment during pregnancy ranges from 26% to 100% (4, 26, 29–33, 37, 38), but apart from the study with the lowest use (33), all others state percentages above 75%. Studies evaluating treatment frequency covered the laboratory non-criteria (4 studies), seronegative, single-positive, and incomplete laboratory (3 studies each), and clinical non-criteria (2 studies) subgroups of NC-APS. Detailed results are presented in Table 3.

Treatment and pregnancy outcomes

In the field of pregnancy morbidity, in the 10 studies reporting a statistical comparison of the pregnancy outcomes of NC-APS versus definite APS, 7 found similar outcomes, including successful pregnancies/live births (1,171 participants) (29, 30, 32, 33, 35, 37, 38). The remaining three (1,830 participants) (4, 26, 31) even described worse outcomes/increased complications at least in some subset of NC-APS in comparison with definite APS: in one the rate of successful pregnancies was lower in women with SN-APS (38.2% vs 50.2%) (4); in a large European registry, even though the rate of live births were similar, obstetric complications occurred in 470 of 640 pregnancies (73.4%) in NC-APS and in 651 of 1000 pregnancies (65.1%) in definite APS (26); and in a study evaluating patients with non-criteria aPL (AhPL isotypes) live births occurred in 53.9% of patients versus 100% in definite APS patients, even though they all were submitted to treatment (31). These studies evaluating pregnancy outcomes covered the laboratory non-criteria and seronegative (4 studies each), single-positive, and incomplete laboratory (3 studies each), and clinical non-criteria (2 studies) subgroups of NC-APS. A summary of these results is available in Table 3.

Regarding the effects of treatment on pregnancy outcomes, 5 studies described an improvement of live births in both NC-APS and definite APS with treatment (26, 32, 33, 37, 38), including patients of the laboratory non-criteria (3 studies), seronegative and incomplete laboratory (2 studies each), and single-positive (1 study) subgroups of NC-APS. In a large European retrospective study analysing LNC-APS patients, the cumulative incidence of adverse obstetrical events was significantly improved in treated patients versus untreated ones, but no difference was found between those receiving aspirin or aspirin/LMWH combination (38). Another study devoted to LNC-APS patients also revealed this finding, with aspirin/LMWH combination being used less frequently in NC-APS, whereas the number of pregnancies with favourable outcome was similar to that of definite APS (33). This lack of difference in pregnancy outcomes between women treated with combination therapy and those receiving aspirin monotherapy was also present in a study including CNC-APS patients (29).

Discussion

This systematic review points towards the absence of marked differences between non-criteria and definite APS in most of the

TABLE 3 Summary of the main statistical comparisons of the included studies[1].

Study	Mekinian,2012 (32)	Rodriguez-Garcia,2012 (4)	Ofer-Shiber,2015 (34)	Mekinian,2016 (33)	Omar,2018 (35)	Signorelli,2017 (36)	Fredi, 2018 (29)	Shi, 2018 (39)	Alijotas-Reig, 2019 (26)	Ferreira, 2020 (27)	Li, 2020 (30)	Lo, 2020 (31)	Abisror, 2020 (38)	Yang, 2021 (37)
Type of NC-APS Parameter	IncompleteAPSSN-APS	SN-APS	IncompleteAPS	LNC-APSSN-APS	SN-APS	Single-positive	CNCAPS	SN-APS	Incomplete APSCNC-APSSingle-positive	SN-APS	Incomplete APSLNC-APSSingle-positive	LNC-APSSingle-positive	LNCAPS	Expert consensus[2]
Vascular thrombosis										[3]				
Venous thrombosis								[4]						
Arterial thrombosis								[4]						
Thrombosis recurrence														
Obstetric morbidity														
≥3 spontaneous abortion <10w														
Fetal loss >10w														
Prematurity <34w														
Treatment frequency (thrombotic APS)														
Treatment frequency (obstetric APS)							[5]						[6]	
Pregnancy outcomes (obstetric APS)									[7]			[8]	[9]	

[1]Yellow boxes represent studies where no differences were found in the evaluated outcome between NC-APS and definite APS patients; green boxes those where the outcome was more frequent in definite APS; red those where the outcome was more frequent in NC-APS; grey boxes represent studies where the specific variable was not evaluated.
[2]Definition according to the Expert consensus on diagnosis and management of obstetric antiphospholipid syndrome of the Chinese Medical Association Society of Perinatal Medicine. The full-text article with the exact definition was not obtainable.
[3]Only triple-positive individuals in the definite APS group.
[4]But simultaneous presence of arterial and venous thrombosis was more common in SN-APS (p=0.012).
[5]Higher use of aspirin/LMW combination in definite APS.
[6]No difference in the frequency of treated pregnancies and use of aspirin/LMWH combination, but higher rate of therapeutic LMWH dose use in definite APS and of aspirin monotherapy in SN-APS.
[7]Live birth rate was similar, but more pregnancy complication occurred in NC-APS (p<0.001).
[8]LNC-APS.
[9]Incomplete laboratory APS.
APS, Antiphospholipid Syndrome; LMWH, Low-molecular-weight heparin; NC-APS, Non-criteria antiphospholipid syndrome.

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evaluated parameters related to clinical manifestations, therapy, and clinical outcomes.

Regarding thrombotic manifestations, the majority of studies found no significant difference regarding the prevalence of arterial/venous thrombosis or thrombosis recurrence. Studies evaluating the occurrence of thrombosis were conducted mainly on seronegative and laboratory non-criteria patients. Conversely, most obstetric studies included an incomplete laboratory APS group (i.e., low titre aPL). This reinforces the previously described notion that low aPL titres, such as those seen in incomplete laboratory APS patients, seem to be particularly implicated in pregnancy morbidity rather than in vascular thrombosis (40, 41). In the specific case of SN-APS, there was the additional suggestion of no difference in thrombosis recurrence.

Regarding obstetric manifestations, most studies also displayed no significant difference in their prevalence between definite and NC-APS; the fact that most studies, including those with most participants (26, 38), focus mainly on obstetric NC-APS may hint a predominantly obstetric phenotype in non-criteria patients.

The review on treatment regimens revealed, firstly, the paucity of data on thrombotic APS, with practically all studies focusing on obstetric patients. An interesting finding was the fact that, despite the absence of formal recommendations, many obstetric NC-APS patients are already treated in a similar fashion to those with definite APS, with no significant difference found in the global prevalence of pregnancy treatment in nearly all studies. This may reflect either the tendency of clinicians to offer treatment when faced with adverse pregnancy outcomes or the eagerness of patients to take them despite the unproven benefits (22). These situations have been reported to thwart the development of clinical trials involving these patients (42). Nevertheless, a significant number of studies described an improvement in live births after treatment in NC-APS, with three studies additionally signalling the possibility that aspirin monotherapy might be as efficacious as aspirin/LMWH combination in these patients (29, 33, 38). This information adds further data to the notion that there may be benefit in treating at least some subgroups of these non-criteria patients, as supported by a recent study displaying improved outcomes with treatment in low titre patients (43). This is a relevant finding, as evidence in this matter is scarce and previous works from an Italian group found no improvement in pregnancy outcomes with the use of low-dose aspirin in patients with "incomplete" obstetric APS (44, 45). Nevertheless, attention should be given to the fact that, in the included articles, there are variations in the treatments prescribed between different institutions, patient groups (i.e., definite and NC-APS) and even among patients of the same group, partially hampering the analysis of the value of specific therapies and regimens.

The noticeable absence of differences in most studies/parameters between definite and NC-APS could underscore the need for a more active evaluation and follow-up of these individuals as, on many occasions, since they do not fulfil the criteria, are not referred for evaluation, or are assessed and discharged without further surveillance. It also highlights the need to further study these individuals to identify in which subsets of non-criteria patients these similarities are effectively present.

This systematic review has strengths but also significant weaknesses. The low-quality evidence (i.e., heterogeneous study populations and designs) and the fact that many studies did not focus on the research questions as their main objective are important limitations. A fact that may affect the frequency of the different parameters evaluated across the studies is the variable definition of non-criteria patients. For instance, studies that do not exclude the presence of other thrombophilia in NC-APS may overestimate the presence of events in these patients, as they may be related to any of these untested thrombophilia; studies which require the presence of non-criteria manifestations in NC-APS patients may also increase the prevalence of thrombosis in these patients, as some of these features are linked with an increased risk of events (46); and studies which include patients with incomplete laboratory criteria (i.e., low titre or single positive aPL) could hypothetically display higher prevalence of events than seronegative patients. In the case of the global proportion of thrombosis/obstetric patients, differences may be consequential to the recruitment site (e.g., thrombosis clinic, pregnancy clinic) resulting in a higher proportion of recruited obstetric or thrombosis patients. Additionally, the fact that only observational studies with considerable risk of bias were included, particularly in the comparability between groups item of the NOS, demonstrates the lack of high-quality research in this field and calls for caution in the interpretation and extrapolation of the results of the retrieved articles. Furthermore, the absence of appropriate matching in the control group of some articles in variables such as age, gender and type of APS (i.e., obstetric or thrombotic) may add possible confounders and further undermine the comparison between groups. A curious finding is the fact that two of the studies with larger samples (26, 38) displayed more prominent differences in outcomes between groups, what could lead to the idea that with a higher number of participants, more disparities could be found between non-criteria and definite APS. Nevertheless, these were also two studies flagged with a considerable risk of bias. One (26) features, in its inclusion criteria for the NC-APS group, mostly patients with only non-criteria obstetric manifestations - this results automatically in a markedly reduced prevalence of criteria manifestations in comparison with definite APS patients, in contrast with most of the other studies. Additionally, one of the subgroups of patients included in this study features individuals who do not meet clinical nor laboratory criteria for definite APS, a fact that might also partially weaken the comparison between groups. In the second one (38), the authors themselves discuss a possible selection bias due to the employed inclusion criteria, where the need for the presence of at least one pregnancy to be included in the NC-APS group might have significantly undermined the inclusion of thrombotic phenotypes in comparison with the definite APS group.

Despite these limitations, the broad search conducted in various databases with the absence of restrictions in publication date and inclusion of congress abstracts allowed for a comprehensive evidence collection. This deliberately far-reaching strategy was adopted, given the heterogeneity in this subject, to ensure an extensive evaluation of all data available. Additionally, the attempt to compartmentalize NC-APS in different subsets also allowed, in some cases, a clearer analysis of the specific type of patients included in each study.

This systematic review hints that most clinical manifestations (obstetric and vascular/thrombotic) are not markedly different between definite and NC-APS. Furthermore, it suggests that most pregnancies in obstetric NC-APS are already being treated as definite APS, and that pregnancy treatment in NC-APS might carry improved outcomes. Aspirin monotherapy might be sufficient in these patients. These findings imply a potential value in a more active follow-up of these patients and hypothesize a possible benefit in managing at least some subgroups of non-criteria patients in a similar fashion to that of definite APS. Nevertheless, the generalization of these results is undermined by the low-quality evidence available, highlighting the need for well-defined and homogenous NC-APS populations in future studies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

GR participated in the study design, data search and extraction, and data analysis. EF participated in the study design, data search, and extraction. BS-P participated in the study design and data analysis. IR-P, AM, IB, RC, and GE participated in the study design, data search and extractions (as referees). All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.967178/full#supplementary-material

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Circulating immune-complexes of IgG/IgM bound to B2-glycoprotein-I associated with complement consumption and thrombocytopenia in antiphospholipid syndrome

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Background: Antiphospholipid syndrome (APS) is a multisystemic autoimmune disorder characterized by thrombotic events and/or gestational morbidity in patients with antiphospholipid antibodies (aPL). In a previous single center study, APS-related clinical manifestations that were not included in the classification criteria (livedo reticularis, thrombocytopenia, leukopenia) were associated with the presence of circulating immune-complexes (CIC) formed by beta-2-glycoprotein-I (B2GP1) and anti-B2GP1 antibodies (B2-CIC). We have performed a multicenter study on APS features associated with the presence of B2-CIC.

Methods: A multicenter, cross-sectional and observational study was conducted on 303 patients recruited from six European hospitals who

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fulfilled APS classification criteria: 165 patients had primary APS and 138 APS associated with other systemic autoimmune diseases (mainly systemic lupus erythematosus, N=112). Prevalence of B2-CIC (IgG/IgM isotypes) and its association with clinical manifestations and biomarkers related to the disease activity were evaluated.

Results: B2-CIC prevalence in APS patients was 39.3%. B2-CIC-positive patients with thrombotic APS presented a higher incidence of thrombocytopenia (OR: 2.32, p=0.007), heart valve thickening and dysfunction (OR: 9.06, p=0.015) and triple aPL positivity (OR: 1.83, p=0.027), as well as lower levels of C3, C4 and platelets (p-values: <0.001, <0.001 and 0.001) compared to B2-CIC-negative patients. B2-CIC of IgM isotype were significantly more prevalent in gestational than thrombotic APS.

Conclusions: Patients with thrombotic events and positive for B2-CIC had lower platelet count and complement levels than those who were negative, suggesting a greater degree of platelet activation.

KEYWORDS

circulating immune-complexes, antiphospholipid syndrome, complement factors, platelets, thrombocytopenia

Introduction

Antiphospholipid syndrome (APS) is defined as a multisystemic autoimmune disorder characterized by the occurrence of thrombotic events and/or gestational morbidity in presence of persistent antiphospholipid antibodies (aPL). Several clinical forms of the syndrome can be identified: 1) APS associated with other systemic autoimmune diseases (SAD-APS) such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA), 2) APS without other associated diseases (primary APS) and 3) catastrophic APS (patients with thrombosis in multiple sites of the vasculature resulting in multiorgan failure with a high mortality rate) (1, 2). Current APS classification criteria require the presence of at least one clinical and one laboratory criterion (3). Clinical criteria include small vessel, arterial or venous thrombosis in any organ or tissue and/or gestational morbidity. Laboratory criteria consist of the presence of some of the following aPL: lupus anticoagulant (LA), anti-cardiolipin (aCL) or anti-β2-glycoprotein-I (aB2GP1) antibodies of IgM or IgG isotype. Positivity must be confirmed at least 12 weeks after the first measurement.

In addition to the clinical manifestations included in the classification criteria, there are other non-criteria clinical features strongly associated with APS such as heart valve disease (4), neurological manifestations (5), livedo reticularis (6) and thrombocytopenia (7). Similarly, other non-criteria autoantibodies have been associated with APS, highlighting the presence of IgA aB2GP1 (8), anti-domain-I of B2GP1 antibodies (9) and IgG/IgM anti-phosphatidylserin/prothrombin antibodies (aPS/PT) (10). Although these antibodies and clinical manifestations are associated with APS, they were not included in the last 2006 criteria update due to the limited evidence at that time (11).

Persistent presence of aPL in asymptomatic carriers implies a paradoxical situation. Although both the antibodies and the main antigen to which they bind (B2GP1) coexist permanently in circulation, thrombotic events only occur occasionally in aPL carriers (risk of developing a thrombotic event in patients with aPL is 3% per year) (12, 13). To explain this situation, the two-hit hypothesis was proposed: antibodies induce a pro-thrombotic state that is necessary but insufficient to trigger thrombotic events and clotting takes place only in the presence of another facilitating condition (second hit) (14). Vascular lesions, infections, inflammatory factors or other procoagulant conditions (such as surgery and immobility) are factors that have been described as triggers of APS events (15).

It has been described that B2GP1 has several conformations. The open (J-shaped) and circular (closed or O-shaped) forms are the most studied conformations. B2GP1 circulates in blood in >99% in a circular conformation. During certain conditions, such as a systemic inflammation or a strong activation of the innate immune system, it is known that the molecule “opens” in

a J-shape conformation, exposing cryptic epitopes (such as in domain I) which are well-known targets of aPL. Thus, the consequences of a second hit at molecular level would be the activation of the B2GP1 with a conformational change, involving the exposure of previously cryptic epitopes (16, 17). In addition to the pathogenic aPL directed against domain I of B2GP1, there are also other thrombosis-associated aPL that recognize epitopes in domains 3 and 4 of the protein (18, 19), whose pathogenicity has been demonstrated by animal models (20). Recently, using hydrogen/deuterium exchange techniques, it has been described that these epitopes in domains 3 and 4 are also located in hidden areas in the closed conformation of B2GP1, which can only be exposed after the activation of the protein and its conversion into the open form (21).

Several authors have emphasized the need for additional biomarkers to improve the assessment of disease activity in APS patients and to identify patients at risk of suffering such events (22, 23). The presence of immune-complexes formed by IgA aB2GP1 bound to B2GP1 (B2A-CIC) has been described in patients with thrombotic manifestations of APS (24, 25). Their presence during the thrombotic event was a risk factor for the development of acute thrombosis (OR 22.7, 95% CI: 5.06-101.57) (24). Besides, the presence of B2A-CIC was evaluated in patients undergoing a renal transplantation. Pre-transplant presence of B2A-CIC was an independent risk factor for graft thrombosis in the first 6 months post-transplant (HR: 14.75, 95% CI: 9.11-23.89) (26). In addition, in the case of patients undergoing cardiac transplantation, post-transplant incidence of thrombosis was significantly higher in B2A-CIC positive patients (OR: 6.42, 95% CI: 2.1-19.63) (27).

Additionally, the presence of immune-complexes constituted by IgG or IgM aB2GP1 bound to B2GP1 (B2G-CIC and B2M-CIC, respectively) was described in a Serbian cohort of APS patients (28). The prevalence of these immune-complexes overall (B2-CIC) was 19.3% and they were associated with an increased incidence of livedo reticularis, ocular dryness, thrombocytopenia and leukopenia as well as with a decrease in the levels of complement factors C3 and C4. However, this study was performed in a single center with a limited number of patients.

In agreement with these results, Taatjes et al. visualized the formation of immune-complexes using sera from APS patients by high resolution microscopy-based imaging techniques (29). Prior to these studies, a small number of reports were published that suggested the presence of B2-CIC in patients with SLE and APS by heparin affinity chromatography (30, 31). However, the complexity of this technology has hampered its use in the daily clinical practice.

Our study has aimed: 1) to develop an easy to use and reproducible method to detect the presence of B2G and B2M-CIC in patients with suspected APS; 2) to determine the prevalence of B2G and B2M-CIC in a multicenter cohort of APS patients; and 3) to analyze the association of B2G/B2M-CIC presence with signs that indicate pathogenic activity of aPL such as the presence of clinical manifestations and laboratory parameters in order to identify patients having a higher thrombotic risk.

Materials and methods

Study design

A cross-sectional, retrospective and multicenter study was performed to determine the prevalence of B2G and B2M-CIC in an APS patient cohort recruited from six European centers, and their association with APS-related clinical and laboratory parameters.

Patients

A total of 303 patients classified as APS were consecutively recruited from six European hospitals during the year 2019:

1) University Hospital Center Bezanijska Kosa, Internal Medicine and Cardiology Units (Belgrade, Serbia) (N=119).

2) National Institute of Geriatrics, Rheumatology and Rehabilitation, Rheumatology Unit (Warsaw, Poland) (N=36)

3) ASST Spedali Civili di Brescia, Rheumatology and Clinical Immunology Unit (Brescia, Italy) (N=38)

4) San Giovanni di Dio Hospital, Rheumatology Unit (Florence, Italy) (N=15)

5) San Giovanni Bosco Hospital, Nephrology and Dialysis Unit (Torino, Italy) (N=66)

6) Hospital Universitario 12 de Octubre, Immunology Department (Madrid, Spain) (N=29).

Inclusion criteria. All patients were examined at their origin center by physicians specialized in Internal Medicine, Rheumatology, Immunology, Neurology, Cardiology or Hematology. Those patients with an APS diagnosis who met the revised classification criteria (both clinical and laboratory criteria) were enrolled in the study (3). Patients were differentiated according to the type of APS event they suffered: 1) isolated thrombotic APS, 2) isolated gestational morbidity, 3) mixed APS (patients who have suffered both thrombotic and gestational manifestations), 4) total thrombotic APS (patients with some thrombotic event, that is, isolated thrombotic and mixed APS) and 5) total gestational morbidity (patients with isolated gestational morbidity and mixed APS).

All SLE-diagnosed patients met the American College of Rheumatology classification criteria (32, 33). Disease activity was assessed at the time of study enrolment using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and only patients with a stable disease were included.

Laboratory and clinical data were obtained from the patient's clinical records at their respective center. The presence of APS clinical criteria, systemic autoimmune diseases, comorbidities (arterial hypertension, diabetes, smoking, dyslipidemia), treatments, laboratory parameters and a series of clinical manifestations associated with APS were included in the data collection form sent to all participating centers.

Exclusion criteria included chronic or acute infections, marked renal/hepatic impairment, presence of present/treated malignancy or patients younger than 18 years.

Laboratory measurements

LA, aCL and aB2GP1 antibodies (of IgG and IgM isotypes) were evaluated at the corresponding centers where patients were recruited, using serum (aCL and aB2GP1 antibodies) or plasma samples (LA) at the same time prior to inclusion or in the blood analysis at enrolment in the study (described in Supplementary Methods). The cutoff used for each measurement calculated based on the 99th percentile of a healthy population or following the manufacturer's guidelines was specific to the local laboratory where quantification was made.

LA evaluation was performed by coagulation assays according to the recommendations of the International Society on Thrombosis and Hemostasis (ISHT) (34). It was based on the prolongation of clotting time by two screening tests (activated partial thromboplastin time or dilute Russell viper venom time), the failure to correct this time by mixing the patient's sample with a normal donor and the correction of clotting time after addition of phospholipids excess. LA tests were performed while patients were not receiving anticoagulant therapy.

After aPL evaluation, samples were stored at -20°C and sent to the Autoimmunity Laboratory at Hospital Universitario 12 de Octubre (Madrid) for the quantification of B2G and B2M-CIC.

Complement factors C3 and C4 were determined by nephelometry using the Beckman Coulter IMMAGE Immunochemistry System (Beckman Coulter Inc. Pasadena, CA, USA). The normal range for C3 levels was 88-225 mg/dl and for C4 levels 12-75 mg/dl. Quantitative detection of human C3a and C5a was performed in 51 patients using the commercial kits Human C3a ELISA and Human C5a ELISA (Invitrogen, ThermoFisher Scientific, Waltham, USA).

Quantification of B2G and B2M-CIC

B2G and B2M-CIC measurement was performed by ELISA. In order to achieve a stable calibration system for transforming optical densities (OD) values into Units/ml, recombinant B2-CIC molecules were generated by transduction of human embryonic kidney cells (HEK293) with lentiviral vectors. The molecules consisted of chimeric proteins containing the Fc region of the corresponding immunoglobulin isotype fused to B2GP1. For the IgG-fused B2GP1 calibrator, a cDNA fragment of human IgG1 that included the hinge region, the constant domains 2 and 3 and the stop codon was incorporated (hinge and Fc regions). For the B2GP1-IgM chimeric protein, the same procedure was performed. In this case, the cDNA encoding the constant domains 2, 3 and 4 of the mu-heavy chain was fused (IgM does not have a hinged region). The final products were tested to ensure that the immunogenicity of each individual proteins (B2GP1 and immunoglobulins heavy chain) was preserved. DNA encoding the calibrator was incorporated into lentiviral vectors, packaged and transduced into HEK293T cells. The proteins were purified from the culture supernatant of recombinant cell lines with stable expression. The procedures for B2G and B2M-CIC production are currently under patent (ES2727261).

Briefly, 96-well Nunc MaxiSorp™ plates (Invitrogen, ThermoFisher Scientific, Waltham, USA) were coated overnight at 4°C with a mouse anti-human B2GP1 monoclonal antibody (ApoH mAb H219, MabTech, Nacka Strand, Sweden) at 2 µg/ml in PBS pH 7.4 for the quantification of B2G and B2M-CIC. Plates were washed 3 times (with PBS-0.1% Tween20), blocked with PBS containing 2% skim milk and 2% polyvinylpyrrolidone (Sigma-Aldrich, Merck KGAA, Darmstadt, Germany) for 1 hour at room temperature (RT) and subsequently washed. Serum samples were diluted at 1:200 in a diluent solution (PBS-0.1% Tween20-0.5% skim milk-0.5% PVP) and serial dilutions were performed for B2G and B2M-CIC calibrators. Diluted samples/blank/calibration curves were dispensed (100 µl) and incubated for 1.5 hours at RT. Plates were washed and incubated for 30 minutes at RT with an anti-human IgG (Anti-human IgG (Fc specific)-Peroxidase antibody, Sigma-Aldrich) or an anti-human IgM antibody (Anti-human IgM (µ-chain specific)-Peroxidase antibody, Sigma-Aldrich) for the detection of B2G and B2M-CIC, respectively. The reaction was revealed by incubation with TMB for 30 minutes (Invitrogen, ThermoFisher Scientific, Waltham, USA) and the addition of 0.344 M sulfuric acid. OD values were measured at 450 nm.

In each assay, sera from three B2-CIC-positive patients (with low, medium and high values) were incorporated as positive controls, as well as sera from three negative controls. The concentration of B2G and B2M-CIC (U/ml) of each patient was obtained by interpolating the optical densities values with the corresponding calibration curve. The suitability of calibration curves was verified by the concordance of the values obtained for the sera with known B2-CIC levels. B2G-CIC and B2M-CIC levels higher than 21 U/ml were considered positive, based on the 99th percentile of a healthy population (213 blood donors from Spain, Italy and Poland). The procedure was performed on the Triturus® Analyzer (Diagnostics Grifols, S.A. Barcelona, Spain).

Evaluation of antiphospholipid immune-complexes by classical methodologies

To confirm that B2-CIC-positive patients detected with the described capture ELISA were also positive by other traditional immune-complexes detection methodologies, six patients with thrombotic APS and positive for B2G-CIC, as well as two negative controls, were studied using Polyethylene Glycol 6000 (PEG-6000) to precipitate immune-complexes, if present.

Immune-complexes were isolated from serum samples by a modification of the Digeon method (35). Briefly, 100 µl of serum were added to 3.9 ml of 3.5% (w/v) of PEG-6000 (ThermoFisher Scientific) in 0.1 M borate buffer pH 8.4. It was incubated for 24 hours at 4°C and the precipitate was pelleted by centrifugation for 15 minutes at 2500 g. The pellet was redissolved in 1 ml of 0.015M PBS, pH 7.4 for 5 minutes at 37°C by gentle shaking. The redissolved immune-complexes were tested by the B2-CIC detection ELISA. Three Nunc MaxiSorp™ plates (Invitrogen, ThermoFisher Scientific) were coated overnight at 4°C with an anti-human B2GP1 monoclonal antibody (ApoH mAb H219, MabTech, Nacka Strand, Sweden) at 2 µg/ml in PBS pH 7.4. Plates were washed 3 times (with PBS-0.1% Tween20), blocked with PBS containing 2% skimmed milk and 2% polyvinylpyrrolidone (Sigma-Aldrich, Merck KGAA, Darmstadt, Germany) for 1 hour at RT and subsequently washed. Polyethylene glycol precipitates from patients and controls were added to each of the three plates and incubated for 1 hour at RT. Plates were washed and revealed using the following secondary antibodies:

Plate 1. Anti-human IgG (Fc-specific)-Peroxidase-antibody (Sigma-Aldrich).

Plate 2. Anti-human C1q antibody conjugated to peroxidase (BioRad, Hercules, CA, USA).

Plate 3. Anti-human ApoH/B2GP1-HRP conjugated (Novus Biologicals, Briarwood, CO, USA).

All plates were incubated for 45 minutes at RT and washed three times. The colorimetric reaction was achieved by incubation with TMB for 30 minutes (Invitrogen, Thermofisher) and the addition of 0.344 M sulfuric acid. OD values were measured at 450 nm.

Immune-complexes stability

To demonstrate that B2-CIC are stable and do not form *de novo* during the detection assay, total IgG from the previous six B2G-CIC-positive patients and two negative controls were purified using Dynabeads Protein G (Invitrogen, ThermoFisher Scientific), following the manufacturer's recommendations. Eluted immunoglobulins were adjusted to a concentration of 5 mg/dl in PBS pH 7.4, which is equivalent to a 1:200 serum dilution (the same dilution used in the assay to quantify B2-CIC).

Nunc MaxiSorp™ plates (Invitrogen, ThermoFisher Scientific) were coated overnight at 4°C with the anti-human B2GP1 monoclonal antibody (ApoH mAb H219, MabTech, Nacka Strand, Sweden) at 2 µg/ml in PBS pH 7.4. Plates were washed three times and blocked. After subsequent washes, 100 µl of purified human B2GP1 (MabTech, Nacka Strand, Sweden) at 10 ng/ml in PBS were added and incubated for 1 hour at RT. For each patient/control, three conditions were used: 1) problem well, in which 100 µl of the purified IgG diluted equivalently to a 1:200 serum dilution were added; 2) positive control and 3) negative control wells, to which 100 µl of PBS were added. After the incubation for 1 hour at RT, plates were washed. In the problem and negative control wells, 100 µl of an anti-human IgG (Fc-specific)-Peroxidase antibody (Sigma-Aldrich) were added. To the positive control wells, 100 µl of an anti-human ApoH/B2GP1-HRP conjugated (Novus Biologicals, Briarwood, CO, USA) were dispensed. Plates were incubated for 45 minutes and washed three times. The reaction was revealed by incubation for 30 minutes with TMB (Invitrogen, ThermoFisher Scientific) and the addition of 0.344 M sulfuric acid. OD values were measured at 450 nm.

In addition, the capacity of purified IgG from patients to recognize B2GP1 in the open conformation was assessed by a commercial ELISA (Orgentec Diagnostika GmbH, Mainz, Germany). IgG concentration was adjusted to be treated as a 1:100 dilution of serum (manufacturer's recommended dilution).

Definitions

Thrombotic event: small vessel, arterial or venous thrombosis in any organ or tissue. The diagnosis must be confirmed by validated objective criteria, such as imaging techniques (3).

Gestational morbidity: death of a morphologically normal fetus, premature delivery or spontaneous abortions defined according to the international classification criteria for APS (3).

Triple aPL positivity: presence of the three laboratory markers associated with APS in the same patient (LA, aCL and aB2GP1 antibodies) (3).

Quadruple aPL positivity: patients with triple aPL positive who are also positive for B2-CIC.

Arterial hypertension: systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg, recorded on different days during evaluations (with evidence of at least 2 readings) or use of antihypertensive medication.

Diabetes mellitus: hyperglycemia resulting from defects in insulin secretion, insulin action, or both (at least 7% of glycated hemoglobin or use of medication).

Dyslipidemia: serum cholesterol concentrations >220 mg/dL, LDL >130 mg/dL, triglycerides >150 mg/dL or use of medication in patients with a history of dyslipidemia.

Smoking: was considered in active or former smokers (individuals who quit smoking less than 6 months ago).

Thrombocytopenia: number of platelets <150,000 per microliter of blood.

Systemic lupus erythematosus (SLE): systemic autoimmune disease whose initial diagnosis was based on the 1997 ACR classification criteria (at least 4 of the 11 criteria were present) (32, 33). These patients would also meet the 2018 ACR/EULAR criteria.

Ethical issues

This study was conducted in accordance with the Ethical Principles for Medical Research of the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of the Hospital Universitario 12 de Octubre (Reference numbers 18/009 and 18/182). Approval for the enrolment of the patients and sample shipment to the Hospital Universitario 12 de Octubre was obtained from the Ethics Committees of each center involved. An informed consent was also obtained from all patients enrolled in the study. Before the samples were sent, an anonymous code was assigned to each patient and serum sample at the hospital of origin to guarantee the anonymity. Anonymized clinical and analytical data from each center were integrated into a single database.

Statistical methods

Clinical and demographic characteristics of patients were described by absolute frequency and percentage or median with the corresponding interquartile range (IQR).

Associations between qualitative variables were performed using Pearson's X^2 test or Fisher's exact test, as appropriate. Wilcoxon-Mann-Whitney test (Mann-Whitney U-test) was used for comparisons of scaled and qualitative variables with two categories and Kruskal-Wallis's test for comparisons of qualitative variables with more than two categories.

Odds ratio (OR) was used to measure the strength of association between the presence of a risk factor and a given outcome. The 95% confidence interval (95% CI) of OR was calculated by logistic regression or Miettinen-Nurminen method, as appropriate (36).

The box and whisker plots represent the values from the lower to upper quartile (from 25 to 75 percentile) in the central box. The median is represented as an inner line in the box. Probabilities <0.05 were considered significant.

Statistical analysis of data was performed using MedCalc Statistical Software version 19.5 (MedCalc Software, Ostend, Belgium).

Results

APS patients' characteristics and prevalence of B2-CIC

A total of 303 patients classified as APS were included in the study from six European hospitals: Belgrade (N=119), Brescia (N=38), Florence (N=15), Madrid (N=29), Torino (N=66) and Warsaw (N=36). The median age of the cohort was 47 years (IQR: 38.0-57.0) and 220 patients (72.6%) were women (male: female ratio of 1:2.7) (Supplementary Table S1).

Clinical presentation. Thrombotic events alone were found in 196 patients (64.7%), 67 (22.1%) only had gestational morbidity and 40 (13.2%) had both thrombotic and obstetric manifestations. Of the total number of patients, 138 (45.5%) had SAD-APS (mainly associated with SLE, N=112, 37.0%) and 165 had primary APS (54.5%). In addition to SLE, other systemic autoimmune diseases present were RA (N=7), Sjögren's syndrome (N=9) and systemic sclerosis (N=3). The main

Table 1 Distribution of positive antiphospholipid antibodies and B2G/B2M-CIC in the 303 APS patients.

ANTIPHOSPHOLIPID ANTIBODIES	MULTICENTER COHORT OF APS PATIENTS (N=303)	
	Positive patients (N)	%
IgG aB2GP1	146	48.2
IgM aB2GP1	149	49.2
IgG aCL	151	49.8
IgM aCL	132	43.6
LA	220	72.6
B2G-CIC	35	11.6
B2M-CIC	98	32.3
B2-CIC	119	39.3
Triple aPL positivity	134	44.2
Quadruple aPL positivity	59	19.5

aB2GP1, anti-β2-glicoproteína-I antibodies; aCL, anti-cardiolipin antibodies; LA, lupus anticoagulant; B2G-CIC, immune-complexes formed by B2GP1 and IgG aB2GP1 antibodies; B2M-CIC, immune-complexes formed by B2GP1 and IgM aB2GP1 antibodies; B2-CIC, presence of B2G and/or B2M-CIC.

clinical characteristics of the cohort are shown in Supplementary Table S2.

Presence of aPL. Of the total patients, 146 (48.2%) were positive for IgG aB2GP1, 149 (49.2%) for IgM aB2GP1, 151 (49.8%) for IgG aCL and 132 (43.6%) for IgM aCL antibodies. LA was the most prevalent aPL, with 220 positive patients (72.6%) (Table 1). In relation to the presence of B2-CIC, 119 patients (39.3%) were positive for some B2-CIC; 35 (11.6%) were positive for B2G-CIC, 98 (32.3%) for B2M-CIC and 14 (4.6%) were positive for both (Table 1). The presence of B2-CIC was not detected in the serum sample in the remaining 184 patients (60.7%).

According to Guilford's Rule of Thumb (37), correlations between the levels of aPL and B2-CIC showed a low-grade relationship in the case of B2G-CIC and IgG aCL (p=0.001, r=0.20) and IgG aB2GP1 antibodies (p<0.001, r=0.25). In patients with B2M-CIC, a moderate relationship with IgM aCL antibody levels (p<0.001, r=0.44) and a negligible relationship with IgM aB2GP1 antibodies (p=0.005, r=0.16) were observed.

Patients were differentiated according to their APS type. The following number of patients was positive for B2-CIC: 73/196 (37.2%) patients with isolated thrombotic APS, 35/67 (52.2%) with isolated gestational morbidity, 11/40 (27.5%) with mixed APS (both thrombotic and gestational manifestations), 84/236 (35.6%) of total thrombotic APS patients and 46/107 (43.0%) of total gestational APS patients (Supplementary Table S3).

Clinical manifestations and laboratory parameters in patients positive for B2-CIC

Based on the presence or absence of B2-CIC (B2G and/or B2M-CIC), no differences were observed in distribution of sex, age, disease duration or proportion of primary APS between both groups. Regarding APS events, a higher percentage of women with isolated gestational morbidity positive for B2-CIC was observed compared to those who were negative (29.4% vs. 17.4%, p=0.014, OR: 1.98, 95% CI: 1.14-3.43) (Table 2).

No differences were observed in the incidence of clinical manifestations associated with APS from different medical specialties, except for a higher incidence of heart valve thickening and dysfunction in B2-CIC-positive patients compared to those who were negative (4.2% vs. 0.5%, p=0.036, OR: 8.03, 95% CI: 1.22-52.45) (Table 2).

In terms of laboratory parameters, although mean C3 and C4 values were within the normal range in both groups, the levels were significantly lower in B2-CIC-positive patients compared to negative patients: C3 levels (median: 113.0 mg/dL, IQR: 92.5-138.0 vs. median: 137.0 mg/dL, IQR: 109.0-167.0, p<0.001, Hodges-Lehmann median difference: 23.0, 95% CI: 13.0-33.0) and C4 levels (median: 20.6 mg/dL, IQR: 14.4-28.1 vs. median: 27.8 mg/dL, IQR: 18.3-37.7, p<0.001, Hodges-Lehmann median difference: 6.39, 95% CI: 3.10-9.60) (Figure 1; Table 2). Levels of complement activation products C3a and C5a were measured in a sample of 51 patients. B2-CIC-positive patients had higher C3a levels compared to the negative ones (mean: 1.98 ng/mL, IQR: 0.87-5.20 vs. mean: 0.82 ng/mL, IQR: 0.30-4.47), but these differences were very close to reaching significance (p=0.059). No differences were observed in the mean levels of C5a between B2-CIC-positive and negative patients (1.73 ng/mL vs. 1.87 ng/mL, respectively, p=0.823).

Additionally, a significant decrease in platelet count was observed in patients positive for B2-CIC compared to the negative ones (median: 216.0 $x10^3$/µL, IQR: 158.8-260.0 vs. median: 234.5 $x10^3$/µL, IQR: 198.0-277.0, p=0.022, median Hodges-Lehmann difference: 23.0, 95% CI: 4.0-44.0). However, this was not associated with a higher incidence of thrombocytopenia in the total cohort (Table 2).

Furthermore, a higher prevalence of IgG aCL antibodies was observed in B2-CIC-positive patients than in those negative (57.1% vs. 45.4%, p=0.038, OR: 1.64, 95% CI: 1.03-2.61), in addition to a higher prevalence of IgM aCL antibodies (52.1% vs. 38.0%, p=0.015, OR: 1.78, 95% CI: 1.12-2.86) (Table 2).

Evaluation of antiphospholipid immune-complexes by classical methodologies

To demonstrate that immune-complexes precipitated by PEG-6000 were formed by B2GP1, a capture ELISA of the precipitates was performed. Six patients with thrombotic APS and positive for B2G-CIC were tested, as well as 2 blood donors as controls. Polyethylene glycol precipitates from all six patients contained B2GP1 (mean: 2.357 OD vs. 0.096 OD in controls). In addition, the presence of IgG and the incorporation of complement factor C1q into B2-CIC was confirmed in all six precipitates but not in controls (mean: 1.113 OD vs. 0.178 OD in controls) (Supplementary Figure S1).

Immune-complexes stability

The same six APS patients positive for B2-CIC and the two controls evaluated by PEG-6000 were also studied to determine whether new B2-CIC could be formed during the time of the assay. We observed that purified IgG from B2-CIC-positive patients was unable to bind to the purified human B2GP1 (>99% in a closed conformation in plasma), with OD values similar to those of the negative control (mean: 0.020 OD and 0.026 OD, respectively). However, the anti-human B2GP1 monoclonal antibody used as a positive control was able to detect the presence of the protein in the assay (mean: 2.726 OD).

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TABLE 2 Clinical, demographic and laboratory parameters of the 303 APS patients grouped according to the positivity for B2-CIC.

CONDITION	B2-CIC positive N=119, 39.3%		B2-CIC negative N=184, 60.7%		p-value	OR/Hodges-Lehmann median difference (95% CI)
	N/median	%/IQR	N/median	%/IQR		
Age (years)	46.0	36.3-56.8	48.0	39.0-57.0	0.533	
Sex (women)	92	77.3	128	69.6	0.141	
Disease duration (years)	5.0	2.0-9.0	6.0	2.0-11.0	0.259	
Systemic lupus erythematosus	42	35.3	70	38.0	0.628	
Diabetes mellitus	7	9.1	15	13.0	0.458	
Arterial hypertension	23	19.3	39	21.2	0.694	
Dyslipidemia	24	20.2	34	18.5	0.716	
Active smoker	9	7.6	23	12.5	0.173	
Primary APS	64	53.8	101	54.9	0.850	
SAD-APS	55	46.2	83	45.1	0.850	
Catastrophic APS	1	0.8	7	3.8	0.154	
Isolated gestational morbidity	35	29.4	32	17.4	**0.014**	1.98 (1.14-3.43)
Isolated thrombotic APS	73	61.3	123	66.8	0.329	
Inf. extr. deep vein thrombosis	35	29.4	58	31.5	0.521	
Superficial thrombophlebitis	19	16.0	23	12.5	0.394	
Sup. extr. arterial thrombosis	1	0.8	8	4.3	0.083	
Acute myocardial infarction	6	5.0	8	4.3	0.779	
Stroke	22	18.5	42	22.8	0.366	
Pulmonary embolism	8	6.7	21	11.4	0.176	
Chorea	3	2.5	3	1.6	0.683	
Epilepsy	4	3.4	14	7.6	0.143	
Migraine	16	13.4	28	15.2	0.670	
Transient global amnesia	4	3.4	2	1.1	0.216	
Cephalea	3	2.5	6	3.3	1.000	
Unstable angina	1	0.8	6	3.3	0.252	
Chronic cardiomyopathy	0	0.0	2	1.1	0.521	
Vegetations	9	7.6	20	10.9	0.340	
Pseudoinfective endocarditis	4	3.4	8	4.3	0.770	
Valve thickening and dysfunction	5	4.2	1	0.5	**0.036**	8.03 (1.22-52.45)
Primary pulmonary hypertension	1	0.8	0	0.0	0.393	
Secondary pulmonary hypertension	3	2.5	1	0.5	0.304	
Major pulmonary arterial thrombosis	2	1.7	2	1.1	0.647	
Pulmonary microthrombosis	5	4.2	16	8.7	0.133	
Glomerular capillary thrombosis	0	0.0	1	0.5	1.000	
Renal artery trunk lesions	0	0.0	2	1.1	0.521	
Renal vein thrombosis	1	0.8	0	0.0	0.393	
Livedo reticularis	26	21.8	28	15.2	0.141	
Skin ulcerations	7	5.9	11	6.0	0.973	
Pseudovasculitic lesions	12	10.1	23	12.5	0.521	
Superficial cutaneous necrosis	3	2.5	5	2.7	1.000	
Amaurosis fugax	0	0.0	2	1.1	0.521	
Retinal artery thrombosis	3	2.5	1	0.5	0.304	
Optic neuropathy	1	0.8	6	3.3	0.251	
Ophthalmic sicca	12	10.1	18	9.8	0.931	
Thrombocytopenia	32	26.9	35	19.0	0.107	
Autoimmune haemolytic anemia	14	11.8	14	7.6	0.223	

(Continued)

TABLE 2 Continued

CONDITION	B2-CIC positive N=119, 39.3%		B2-CIC negative N=184, 60.7%		p-value	OR/Hodges-Lehmann median difference (95% CI)
	N/median	%/IQR	N/median	%/IQR		
Microangiopathic haemolytic anemia	4	3.4	2	1.1	0.216	
Leucopenia	18	15.1	30	16.3	0.784	
Disseminated intravascular coagulation	2	1.7	4	2.2	1.000	
C3 levels (mg/dL)	113.0	92.5-138.0	137.0	109.0-167.0	**<0.001**	23.0 (13.0-33.0)
C4 levels (mg/dL)	20.6	14.4-28.1	27.8	18.3-37.7	**<0.001**	6.39 (3.10-9.60)
Hypocomplementemia	32	26.9	36	19.6	0.167	
Platelets (x10^3/μL)	216.0	158.8-260.0	234.5	198.0-277.0	**0.022**	23.0 (4.0-44.0)
IgG aCL positive	68	57.1	83	45.4	**0.038**	1.64 (1.03-2.61)
IgM aCL positive	62	52.1	70	38.0	**0.015**	1.78 (1.12-2.86)
IgG B2GP1 positive	62	52.1	84	45.7	0.273	
IgM B2GP1 positive	62	52.1	87	47.3	0.413	
LA positive	87	73.1	133	72.3	0.875	
Triple aPL positivity	59	49.6	75	40.8	0.132	
B2G-CIC positive	35	29.4	Na	Na	Na	
B2M-CIC positive	98	82.4	Na	Na	Na	
Antiplatelet agents	77	64.7	129	70.1	0.299	
Anticoagulants	108	90.8	161	87.5	0.350	
Treated	112	94.1	170	92.4	0.519	

Significant p-values <0.05 are represented in bold.

The ability of purified IgG from the six B2G-CIC-positive patients to recognize the open conformation of B2GP1 was tested using a commercial assay designed to determine aB2GP1 antibodies. Mean levels of IgG aB2GP1 antibodies of patients were 67 U/ml. The lowest value was 23 U/ml, which was above the cut-off point considered by the manufacturer (10 U/ml). These results suggest that patient sera are able to recognize the open form of B2GP1 but not the closed conformation in plasma.

Characteristics of patients with thrombotic APS and B2-CIC

Considering the 236 patients who had suffered thrombotic events (total thrombotic APS, without excluding patients who additionally had some obstetric symptoms), no significant differences were observed in sex, age, disease duration, presence of cardiovascular risk factors, proportion of primary

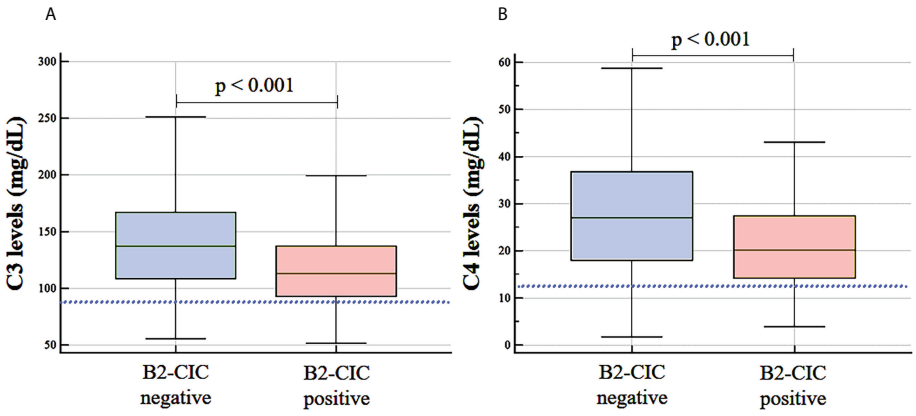

FIGURE 1
Levels of complement factors C3 **(A)** and C4 **(B)** in B2-CIC-positive and negative patients (N=303). The lower limit of the normal range for C3 (88 mg/dl) and C4 levels (12 mg/dl) is represented by dotted lines.

APS or incidence of most APS-related clinical manifestations between positive and negative patients for B2-CIC, except for a higher incidence of heart valve thickening and dysfunction in B2-CIC-positive patients compared to the negative patients (6.0% vs. 0.7%, p=0.015, OR: 9.60, 95% CI: 1.44-62.71) (Supplementary Table S4).

A significant decrease in platelet count was observed in B2-CIC-positive patients in comparison with those who were negative (median: 199.5 x10^3/μL, IQR: 135.0-252.0 vs. median: 235.5 x10^3/μL, IQR: 200.0-288.0, p=0.001, median Hodges-Lehmann difference: 42.0, 95% CI: 16.0-68.0) in addition to a higher incidence of thrombocytopenia (33.3% vs. 17.8%, p=0.007, OR: 2.32, 95% CI: 1.25-4.28) (Figures 2A, B; Supplementary Table S4). Besides, a significant reduction in the complement factors levels was observed in patients positive for B2-CIC compared to the negative ones: C3 levels (median: 109.0 mg/dL, IQR: 91.0-129.0 vs. median: 138.5 mg/dL, IQR: 109.0-167.0, p<0.001, Hodges-Lehmann median difference: 29.0, 95% CI: 18.0-41.0) and C4 levels (median: 18.9 mg/dL, IQR: 13.0-26.8 vs. median: 27.7 mg/dL, IQR: 18.6-37.4, p<0.001, Hodges-Lehmann median difference: 7.90, 95% CI: 4.20-11.88) (Figures 2C, D; Supplementary Table S4).

A higher prevalence of IgG aCL antibodies was observed in B2-CIC-positive patients compared to the B2-CIC negative patients (64.3% vs. 46.1%, p=0.005, OR: 2.18, 95% CI: 1.26-3.79) in addition to a higher incidence of triple aPL positivity (57.1% vs. 42.1%, p=0.027, OR: 1.83, 95% CI: 1.07-3.14) (Supplementary Table S4).

Characteristics of patients with isolated thrombotic APS and B2-CIC

When the 196 patients with isolated thrombotic APS were considered, we observed the same pattern as in the previous group. No differences were observed in age, sex, disease duration, proportion of primary APS or presence of most clinical manifestations associated with APS between B2-CIC-positive and negative patients. However, a higher incidence of heart valve thickening was observed in patients positive for B2-CIC compared to those who were negative (5.5% vs. 0.8%, p=0.047, OR: 7.07, 95% CI: 1.03-47.97) (Table 3).

A decrease in the platelet count was observed in patients with B2-CIC in relation to patients without these (median: 198.0 x10^3/μL, IQR: 130.0-253.3 vs. median: 244.0 x10^3/μL,

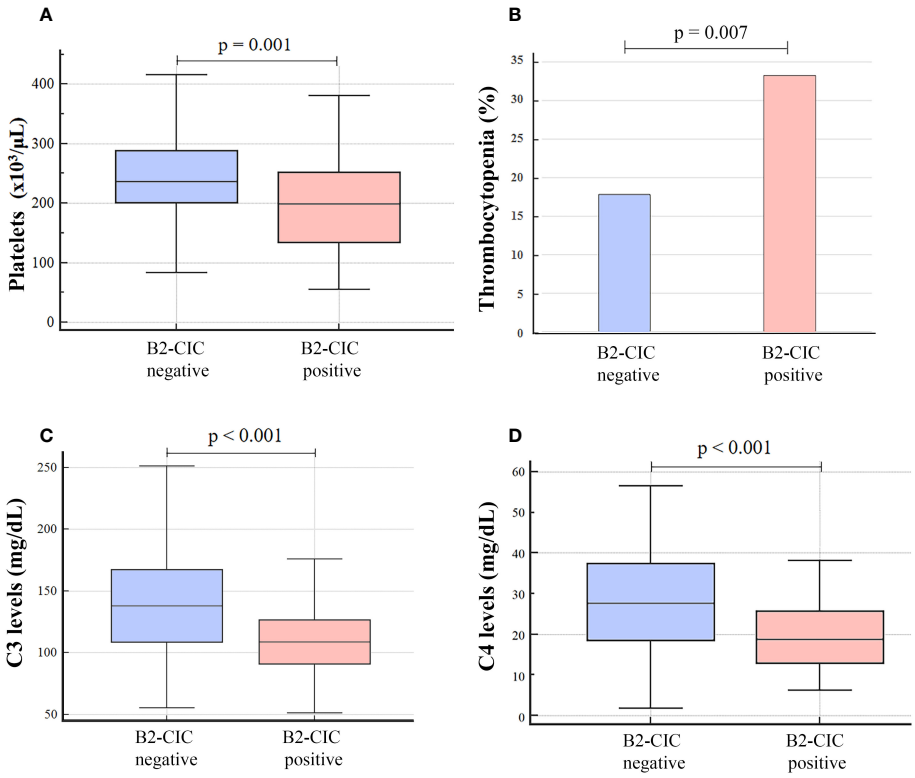

FIGURE 2
Platelet count (**A**), incidence of thrombocytopenia **(B)** and levels of C3 **(C)** and C4 **(D)** in patients with total thrombotic APS, positive and negative for B2-CIC (N=236).

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IQR: 203.3-292.3, p=0.001, Hodges-Lehmann median difference: 46.0, 95% CI: 21.0-76.0) and this was associated with a higher incidence of thrombocytopenia (30.1% vs. 16.3%, p=0.022, OR: 2.22, 95% CI: 1.11-4.44) (Figures 3A, B; Table 3).

Lower levels of complement factors were also observed in patients positive for B2-CIC versus those who were negative: C3 levels (median: 109.0 mg/dL, IQR: 90.0-130.0 vs. median: 135.5 mg/dL, IQR: 108.5-161.5, p<0.001, Hodges-Lehmann median difference: 27.0, 95% CI: 15.0-39.0) and C4 levels (median: 18.8 mg/dL, IQR: 13.0-25.8 vs. median: 26.2 mg/dL, IQR: 17.2-35.5, p=0.001, Hodges-Lehmann median difference: 7.10, 95% CI: 3.30-11.10) (Figures 3C, D; Table 3).

A higher prevalence of IgG aCL antibodies was observed in B2-CIC-positive patients compared to those who were negative (64.4% vs. 46.3%, p=0.011, OR: 2.18, 95% CI: 1.19-3.97), as well as a higher prevalence of triple aPL positivity (58.9% vs. 39.8%, p=0.010, OR: 2.17, 95% CI: 1.20-3.90) (Table 3).

In addition, a lower proportion of B2-CIC-positive patients with isolated thrombotic APS were treated with antiplatelet

Table 3 Clinical, demographic and laboratory parameters of patients with isolated thrombotic APS, positive and negative for B2-CIC (N=196).

ISOLATED THROMBOTIC APS (N=196)

CONDITION	B2-CIC positive N=73, 37.2%		B2-CIC negative N=123, 62.8%		p-value	OR/Hodges-Lehmann Median Difference (95% CI)
	N/median	%/IQR	N/median	%/IQR		
Age (years)	54.0	41.0-61.3	50.0	40.3-58.0	0.205	
Sex (women)	46	63.0	67	54.5	0.243	
Disease duration (years)	5.0	3.0-10.0	5.0	2.0-10.0	0.393	
Systemic lupus erythematosus	30	41.1	51	41.5	0.960	
Primary APS	36	49.3	63	51.2	0.797	
SAD-APS	37	50.7	60	48.8	0.797	
Catastrophic APS	0	0.0	4	3.3	0.299	
Arterial thrombosis	32	43.8	63	51.2	0.319	
Venous thrombosis	43	58.9	65	52.8	0.170	
Inf. extr. deep vein thrombosis	31	42.5	50	40.7	0.455	
Superficial thrombophlebitis	15	20.5	18	14.6	0.286	
Sup. extr. arterial thrombosis	1	1.4	6	4.9	0.239	
Acute myocardial infarction	4	5.5	5	4.1	0.729	
Stroke	20	27.4	36	29.3	0.780	
Pulmonary embolism	7	9.6	13	10.6	0.827	
Valve thickening and dysfunction	4	5.5	1	0.8	**0.047**	7.07 (1.03-47.97)
Thrombocytopenia	22	30.1	20	16.3	**0.022**	2.22 (1.11-4.44)
Hypocomplementemia	24	32.9	28	22.8	0.141	
C3 levels (mg/dL)	109.0	90.0-130.0	135.5	108.5-161.5	**<0.001**	27.0 (15.0-39.0)
C4 levels (mg/dL)	18.8	13.0-25.8	26.2	17.2-35.5	**0.001**	7.10 (3.30-11.10)
Platelets ($x10^3/\mu L$)	198.0	130.0-253.3	244.0	203.3-292.3	**0.001**	46.0 (21.0-76.0)
IgG aCL positive	47	64.4	57	46.3	**0.011**	2.18 (1.19-3.97)
IgM aCL positive	35	47.9	45	36.6	0.100	
IgG B2GP1 positive	43	58.9	56	45.5	0.071	
IgM B2GP1 positive	36	49.3	53	43.1	0.399	
LA positive	57	78.1	88	71.5	0.314	
B2G-CIC positive	26	35.6	Na	Na	Na	
B2M-CIC positive	58	79.5	Na	Na	Na	
Triple aPL positivity	43	58.9	49	39.8	**0.010**	2.17 (1.20-3.90)
Antiplatelet agents	35	47.9	78	63.4	**0.028**	0.51 (0.28-0.93)
Anticoagulants	64	87.7	106	86.2	0.817	
Treated	68	93.2	111	90.2	0.477	

aB2GP1, anti-β2-glicoproteína-I antibodies; aCL, anti-cardiolipin antibodies; LA, lupus anticoagulant; B2G-CIC, immune-complexes formed by B2GP1 and IgG aB2GP1 antibodies; B2M-CIC, immune-complexes formed by B2GP1 and IgM aB2GP1 antibodies. Significant p-values <0.05 are represented in bold.

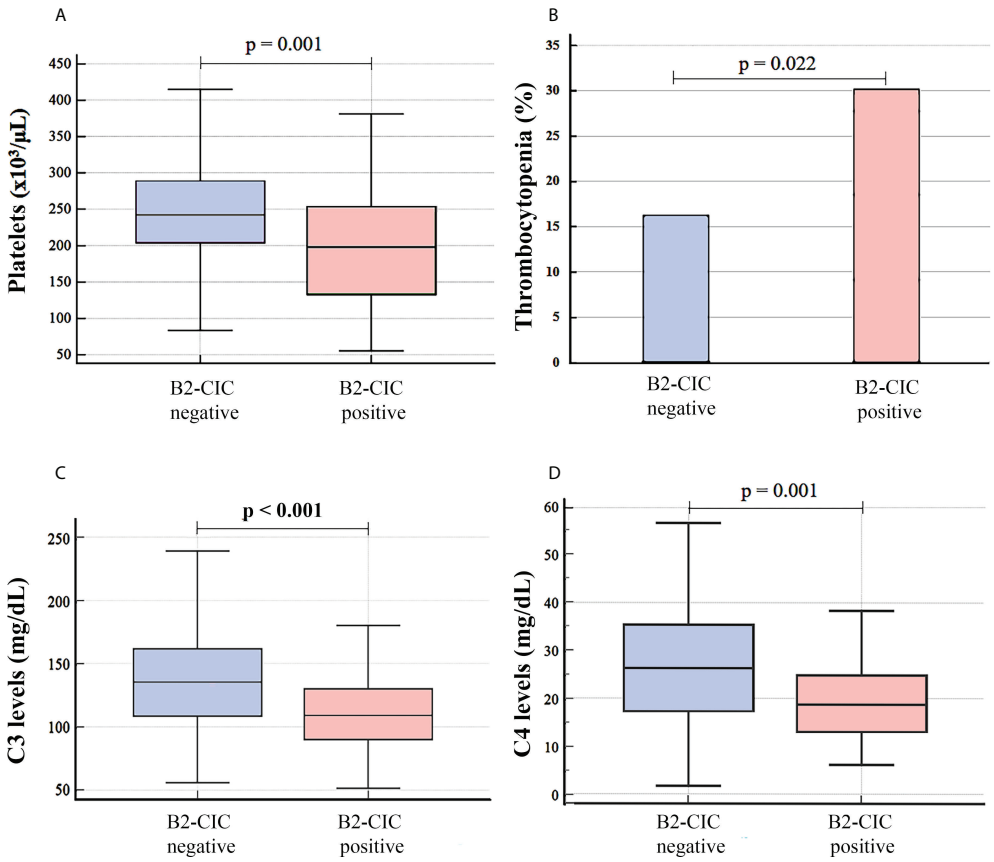

FIGURE 3
Platelet count (**A**), incidence of thrombocytopenia **(B)** and levels of complement factor C3 **(C)** and C4 **(D)** in patients with isolated thrombotic APS, differentiating between B2-CIC-positive and negative patients (N=196).

therapy compared to the negative ones (47.9% vs. 63.4%, p=0.028, OR: 0.51, 95% CI: 0.28-0.93), although no differences in terms of anticoagulant therapy or proportion of treated patients were observed (Table 3).

Characteristics of patients with gestational morbidity and B2-CIC

Taking into account the 107 patients with gestational morbidity (without excluding those who had suffered an additional thrombotic event), no significant differences were observed in the main clinical characteristics or presence of manifestations associated with APS between B2-CIC positive and negative patients. A shorter disease duration (years since the diagnosis) was observed in the case of B2-CIC-positive patients compared to the negative ones (median: 3.0 years, IQR: 1.6-7.3 vs. median: 8.0 years, IQR: 4.0-14.0, p=0.002, Hodges-Lehmann median difference: 3.0, 95% CI: 1.0-6.0).

In the 40 patients with mixed APS (both thrombotic events and gestational morbidity), no differences were observed between B2-CIC-positive and negative patients for the main clinical characteristics and laboratory parameters evaluated. As occurred previously, only a shorter disease duration was observed in B2-CIC-positive patients compared to the negative ones (median: 4.0 years, IQR: 2.0-6.8 vs. median: 8.5 years, IQR: 4.0-17.6, p=0.021, Hodges-Lehmann median difference: 4.5, 95% CI: 1.0-11.9).

Characteristics of patients with isolated gestational morbidity and B2-CIC

In the 67 patients with isolated gestational APS, no significant differences were observed in age, presence of clinical manifestations related to APS, disease duration, laboratory parameters or treatment received between the B2-CIC-positive and negative patients.

Differences between isolated thrombotic and gestational APS patients

To determine whether the previous differences were observed due to the syndrome's clinical presentation rather than the presence of B2-CIC, we compared the previously identified significant variables (p<0.050) between the three main groups of patients according to the clinical events they suffered (independently of B2-CIC positivity): isolated thrombotic APS, isolated gestational morbidity and mixed APS.

Patients with isolated gestational morbidity were younger (median: 39.0 years, IQR: 32.0-44.0) than patients with mixed APS (median: 49.0 years, IQR: 37.5-56.5, p=0.003) and isolated thrombotic APS (median: 51.5 years, 41.0-59.0, p<0.001) (Supplementary Table S5). In addition, a higher proportion of patients with isolated gestational morbidity were treated with antiplatelet agents (91.0%) compared to patients with mixed APS (80.0%, p=0.033) and isolated thrombotic APS (57.7%, p<0.001). This difference was also observed between patients with mixed APS and isolated thrombotic APS (p=0.015) (Supplementary Table S5).

Higher B2M-CIC levels were observed in patients with isolated gestational morbidity (median: 19.0 U/mL, IQR: 8.2-30.8) compared to patients with isolated thrombotic APS (median: 11.0 U/mL, IQR: 5.8-22.1, p=0.019), but not when compared with mixed APS (median: 10.1 U/mL, IQR: 5.9-24.0, p=0.089). No differences were observed between patients with isolated thrombotic and mixed APS (p=0.990). The difference observed in B2M-CIC levels resulted in a higher prevalence of B2M-CIC-positive patients with isolated gestational morbidity (44.8%) in comparison with patients with isolated thrombosis (29.6%, p=0.023) and mixed APS (25.0%, p=0.042) (Supplementary Table S5).

Lastly, higher B2G-CIC levels were observed in patients with isolated thrombotic APS (median: 5.6 U/mL, IQR: 3.1-10.5) compared to patients with isolated gestational morbidity (median: 3.8 U/mL, IQR: 2.0-8.2, p=0.013) and mixed APS (median: 4.2 U/mL, IQR: 2.2-6.0, p=0.015). No difference was observed between patients with mixed APS and isolated gestational morbidity (p=0.750). However, the difference found in the levels did not imply a significant difference in the prevalence of B2G-CIC-positive patients (Supplementary Table S5).

Statistically significant differences were observed in the lower levels of C3, C4 and platelets in B2-CIC-positive patients with isolated thrombotic APS compared to the negative patients as well as in the higher prevalence of thrombocytopenia only when patients were differentiated according to the positivity for B2-CIC (Figure 4; Table 3). These differences were not observed in patients with isolated gestational morbidity or mixed APS (Figure 4).

Finally, patients with B2-CIC and isolated thrombotic APS were compared to B2-CIC-positive patients with isolated gestational morbidity. Patients with thrombotic APS were older than gestational patients (median: 54.0 years, IQR: 41.0-61.3 vs. median: 38.0 years, IQR: 33.3-41.8, p<0.001, Hodges-Lehmann median difference: 15.0, 95% CI: 9.0-19.0) and had a higher prevalence of triple aPL positivity (58.9% vs. 31.4%, p=0.009, OR: 3.13, 95% CI: 1.33-7.34) (Table 4). Regarding complement factors, patients with thrombotic APS had lower C3 levels compared to patients with gestational morbidity (median: 109.0 mg/dL, IQR: 90.0-130.0 vs. median: 132.0 mg/dL, IQR: 100.5-144.8, p=0.028, Hodges-Lehmann median difference: 19.0, 95% CI: 2.0-34.0), in addition to lower C4 levels (median: 18.8 mg/dL, IQR: 13.0-25.8 vs. median: 24.2 mg/dL, IQR: 18.5-31.9, p=0.016, Hodges-Lehmann median difference: 5.70, 95% CI: 1.20-10.30) (Table 4). Additionally, a decrease in the platelet count was observed in patients with thrombosis compared to those with obstetric manifestations (median 198.0 $x10^3$/μL, IQR: 130.0-253.3 vs. median: 239.0 $x10^3$/μL, IQR: 197.5-270.5, p=0.025, Hodges-Lehmann median difference: 36.0, 95% CI: 5.0-67.0), resulting in a higher incidence of thrombocytopenia (30.1% vs. 11.4%, p=0.034, OR: 3.34, 95% CI: 1.05-10.61) (Table 4).

The same statistically significant differences were obtained when B2-CIC-positive patients with total thrombotic APS were compared with B2-CIC-positive patients with isolated gestational morbidity (data not shown).

Discussion

The presence of B2G and B2M-CIC in a cohort of APS patients having different geographical origins has been confirmed in this multicenter study. B2-CIC presence in patients with thrombotic antecedents was significantly associated with a decrease of the platelet count and complement factors C3 and C4 levels as well as with a higher incidence of thrombocytopenia, thus confirming what has been observed in previous single-center studies (26–28).

In APS patients, the development of thrombotic events is not always triggered by the presence of aPL. Therefore, additional biomarkers are needed to support the disease assessment. In regards to this, the presence of B2A-CIC has been described in patients with thrombotic manifestations of APS (24, 25). A prevalence of 39.3% for B2-CIC formed by aB2GP1 antibodies of IgG or IgM isotypes was observed in this multicenter study performed on a cohort of 303 patients with a diagnosis of APS. Patients with B2-CIC and thrombotic APS had a higher incidence of thrombocytopenia, heart valve thickening, and triple aPL positivity compared to the B2-CIC negative patients. Although the mean levels of C3 and C4 in patients with B2-CIC were within the normal ranges, both were significantly lower than in patients

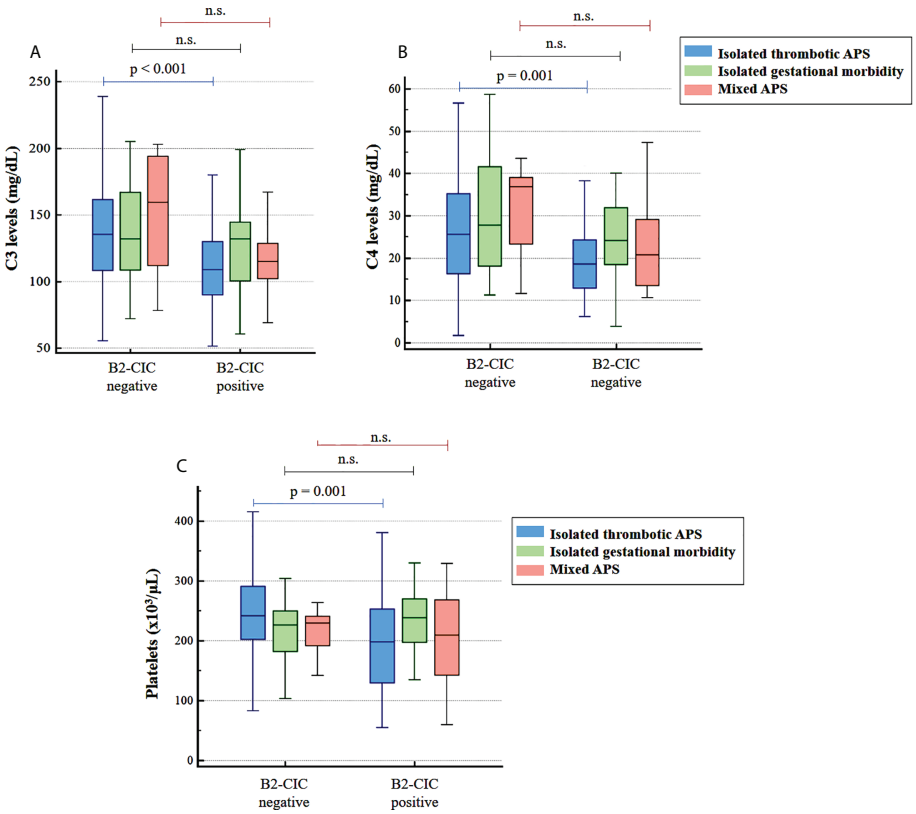

FIGURE 4
Levels of C3 (**A**), C4 **(B)** and platelets **(C)** in patients with isolated thrombotic APS (blue), isolated gestational morbidity (green) and mixed APS (red) according to the positivity for B2-CIC. ns, not significant.

Table 4 Comparison of clinical and laboratory characteristics previously identified with a p-value <0.050 between B2-CIC-positive patients with isolated thrombotic APS and B2-CIC-positive patients with isolated gestational morbidity.

CONDITION	ISOLATED THROMBOTIC APS B2-CIC + (N=73, 37.2%)	ISOLATED GESTATIONAL APS B2-CIC + (N=35, 52.2%)	p-value	OR/Hodges-Lehmann Median Difference (95% CI)
	N (%)/median (IQR)	N (%)/median (IQR)		
Age (years)	54.0 (41.0-61.3)	38.0 (33.3-41.8)	**<0.001**	15.0 (9.0-19.0)
Platelets (x10^3/µL)	198.0 (130.0-253.3)	239.0 (197.5-270.5)	**0.025**	36.0 (5.0-67.0)
Thrombocytopenia	22 (30.1)	4 (11.4)	**0.034**	3.34 (1.05-10.61)
C3 levels (mg/dL)	109.0 (90.0-130.0)	132.0 (100.5-144.8)	**0.028**	19.0 (2.0-34.0)
C4 levels (mg/dL)	18.8 (13.0-25.8)	24.2 (18.5-31.9)	**0.016**	5.70 (1.20-10.30)
Valve thickening and dysfunction	4 (5.5)	0 (0.0)	0.160	
Triple aPL positivity	43 (58.9)	11 (31.4)	**0.009**	3.13 (1.33-7.34)
Disease duration (years)	5.0 (3.0-10.0)	3.0 (1.5-9.0)	0.079	

Significant p-values <0.05 are represented in bold.

without B2-CIC. These differences were not observed in patients with mixed APS or isolated gestational morbidity.

In order to confirm whether the differences found between patients with and without B2-CIC were observed due to the type of APS presented rather than to the presence of B2-CIC, a comparison of the patients was conducted only in regards to the clinical events they suffered from. Patients with isolated gestational morbidity were younger than patients with mixed

and isolated thrombotic APS, coinciding with an obvious younger age for women of fertile age. In addition, they were more frequently treated with antiplatelet therapy compared to the two other groups. This is related to the standard therapy received for treating each type of event, since low-dose aspirin is recommended for non-pregnant women with a history of obstetric APS and also during pregnancy together with heparin, while the baseline treatment for venous and arterial thrombosis is mainly by anticoagulation (VKA) (38). Furthermore, higher levels of B2M-CIC were observed in patients with isolated gestational morbidity compared to patients with isolated thrombotic APS, but not with mixed APS (probably due to the low number of patients in this group). This difference in the levels resulted in a higher prevalence of B2M-CIC-positive patients with isolated gestational morbidity in relation to patients with thrombosis.

It stands out that there was a significantly higher proportion of B2M-CIC-positive patients among those with obstetric APS without a history of thrombosis (isolated gestational APS). This finding is in agreement with the known fact that the IgM isotype is important in obstetric APS: isolated IgM aPL are frequent in obstetric APS and rare in thrombotic APS (39). This reinforces the idea that obstetric and thrombotic APS most likely have a different pathogenesis despite sharing the same antigenic specificity of the antibodies (40).

Under physiological conditions, B2GP1 is only present in the decidual endothelium and placental tissues but not in the endothelium of the rest of the body, where is only detected after an inflammatory stimulus (41, 42). The placental damage found during pregnancy could be explained because the large amount of B2GP1 would be recognized by aPL in both free form and bound to B2GP1, forming B2-CIC. Since IgM is the most efficient isotype activating complement (43), the pathogenic role of B2M-CIC could be mediated by the complement. The role of complement in obstetric APS is well known as the deposition of complement factors has been described in both the placentas of gestational-APS patients and in animal models of this illness. Furthermore, the infusion of complement-blocking molecules has been shown to protect against fetal loss in these pregnant mice APS models (44–46).

On the contrary, although patients with isolated thrombotic APS had higher levels of B2G-CIC compared to patients with isolated gestational morbidity and mixed APS, there were no differences in the prevalence of B2G-CIC-positive patients among the three groups.

Our results show that the type of APS event was not associated to the decrease of platelet and complement factor levels between patients positive and negative for B2-CIC. The comparison of B2-CIC-positive patients with isolated thrombotic APS (as well as patients with total thrombotic APS) and B2-CIC-positive patients with isolated gestational morbidity confirmed that patients with thrombotic manifestations and positive for B2-CIC had lower levels of C3, C4 and platelets as well as an increased incidence of thrombocytopenia and triple positivity compared to patients with obstetric APS. The higher C3a levels in patients with B2-CIC, although not significant, could suggest a tendency towards an increased complement activation in these patients. The nearly significant and non-significant association of C3a and C5a levels with the presence of B2-CIC, respectively, may be due to the short half-life of these complement activation products [a half-life of 30 minutes for C3a and 5 minutes for C5a has been described (47–49)]. Although patients with isolated gestational morbidity had a higher prevalence of B2-CIC, this did not result in an alteration of complement factors or platelets. However, patients with thrombotic APS had a higher complement and platelet consumption, suggesting a higher degree of platelet activation leading to an increase of thrombocytopenia in B2-CIC-positive patients. In the case of complement factors, the consumption was moderate and although there was a decrease in the total levels of C3 and C4, an increased percentage of patients with hypocomplementemia was not found.

A clear correlation was not observed in the evaluation of circulating levels of aPL and B2-CIC. The aPL evaluation assays can only detect the free forms of antibodies but not aPL bounded to their antigen. In this way, B2-CIC-positive patients may have low levels of circulating aPL or even negative results because the antibodies integrated in B2-CIC could not be detected, so the observed aPL levels may be lower than those prior to the B2-CIC formation (30, 50). Only low-affinity antibodies would remain not attached to their antigen and thus could be detected by conventional assays. This could be a paradoxical situation in which the presence of high-affinity antibodies could predominate in patients in whom the detected aPL titers by conventional assays are below the cutoff. Future studies evaluating this possibility would be of interest.

We have verified that purified immunoglobulins from B2G-CIC-positive patients are not able to recognize the purified B2GP1 from human serum (>99% in a circular conformation under physiological conditions). However, these purified immunoglobulins can bind to B2GP1 when it is in an open form (easily detected by commercial assays for the determination of IgG aB2GP1 antibodies), confirming our suspicions that B2-CIC can only be formed after B2GP1 changes its conformation to the open form, a situation that only occurs after its activation. These results are in agreement with Meroni's two-hit hypothesis: the presence of aPL is not sufficient to develop thrombotic events because B2GP1 circulates in a closed conformation, so a second hit that activates the endothelium is needed (14). B2GP1 binds to activated endothelial cells, opens its conformation and exposes hidden epitopes enabling the binding of aPL. The B2GP1-aPL binding is stable and can only dissociate under conditions involving extreme pH shocks or after numerous cycles of freezing and thawing (25). The presence of B2-CIC could indicate that, at some moment, a second hit could have triggered the

conformational change of B2GP1 to the open form, allowing the antigen-antibody binding. In this sense, the presence of B2-CIC maybe could be a surrogate marker for the action of a second hit, triggering the protein opening and allowing aPL binding. This hypothesis has to be confirmed by future studies.

The presence of B2-CIC in APS was described in 1995 in a patient with catastrophic APS by heparin affinity chromatography (51). The use of this challenging technology was hampered in the clinical practice and the presence of B2-CIC has been poorly studied up to date. In this study, we have used an easily reproducible methodology to detect the presence of B2G and B2M-CIC that could be implemented in a simple manner in diagnostic laboratories. Using this method, our group have recently described the presence of B2A-CIC (24, 25), B2G-CIC and B2M-CIC (28) in APS patients. They were associated with an increased risk of thrombosis or presence of clinical manifestations related to APS such as thrombocytopenia and livedo reticularis (26–28).

It is well known that the APS clinical spectrum goes beyond the classification criteria and there are other clinical events strongly associated with APS such as thrombocytopenia (11, 52). In this multicenter cohort, thrombocytopenia is the most frequently observed non-criteria clinical manifestation, with a prevalence of 22% in the total cohort and 33.3% in B2-CIC-positive patients with thrombotic APS. It is similar to the estimated prevalence in other studies (20-53%) (7). In the study of 1000 APS patients performed by the Euro-Phospholipid Project Group, thrombocytopenia was the most frequent non-criteria clinical manifestation observed (29.6%). If clinical classification criteria are also considered, thrombocytopenia would be the second most frequent manifestation found after deep vein thrombosis (53). After a 10-year follow-up of these APS patients, the most common manifestations observed (including both criteria and non-criteria) were thrombocytopenia, livedo reticularis, stroke and gestational morbidity (12). Some authors have proposed that patients with thrombocytopenia and positive aPL represent a subgroup of patients with a pro-thrombotic state that precedes the onset of APS (52, 54, 55). Following the 14th International Congress on Antiphospholipid Antibodies, the task force recommended the inclusion of manifestations such as thrombocytopenia in the revision of classification criteria (56). The role of aPL in the pathogenesis of thrombocytopenia is not clearly understood. It has been postulated that these autoantibodies bind to activated platelets through the interaction of B2GP1 to glycoprotein Ibα (GPIbα) and ApoER2 receptors (57, 58). After the binding of aB2GP1/B2GP1 immune-complexes to these receptors, platelet activation *via* p38MAPK pathway was observed (59, 60). This platelet activation led to a subsequent endothelial cell activation and fibrin generation (61, 62). This mechanism could explain the thrombocytopenia found in patients with B2-CIC. Using murine models of APS, thrombocytopenia is developed as one of the most relevant features of the disease when mice are immunized with purified B2GP1 protein (63, 64).

In APS, the antigen (B2GP1) and the aB2GP1 antibody that recognizes it coexist in blood, but the occurrence of thrombosis is not always induced. Therefore, there must be more than one mechanism involved in the pathogenesis of APS. The presence of circulating B2-CIC could be a new biomarker related to the presence of the protein B2GP1 circulating in an open or "hook" conformation that could be recognized by aPL present in blood due to the exposure of epitopes previously hidden in the closed conformation that were not accessible by aPL (65, 66). The presence of open forms of B2GP1 would imply that aPL could be interacting with the soluble protein but also with this form of B2GP1 associated to membrane receptors such as GPIbα, ApoER2, annexin A2 or toll-like receptors (previously described as B2GP1-associated molecules) (15). The stimulation of these receptors by aPL initiates the activation of endothelial cells and could activate the complement pathway causing inflammatory lesions (66).

The complement system plays an important role in the clearance of pathogens and apoptotic cells and it is an important component of the immune response (67). We have observed lower levels of C3 and C4 in B2-CIC-positive patients with thrombotic APS, suggesting a higher degree of complement consumption. Moreover, it has been confirmed that the detected immune-complexes are formed by B2GP1 and IgG aPL, and they can incorporate complement factor C1q. IgG and IgM antibodies present in B2-CIC could lead to an increased activation of the classical pathway of complement (68, 69) as well as the lectin pathway (70). Clinical and experimental data accumulated in recent years support the role of the complement system as a key factor in the pathogenesis of APS (44, 71). A reduction in C3 and C4 levels (in addition to an increase of their activation products C3a and C4a) has been described in patients with vascular APS (72, 73), suggesting that the decrease in the complement levels is due to their consumption and activation of complement pathway (74). However, no association with serological parameters has been found. Moreover, some studies have described deposits of Ig, C1q and C3 in the heart valves of patients with aPL-associated valvulopathy (75), in renal biopsies of patients with APS nephropathy (76) and in a male patient who underwent bypass surgery after which he developed arterial thrombosis (77). Therefore, in addition to serological consumption, these findings suggest that complement activation also takes place at the tissue level. The critical role of the complement in mediating the damaging effects of aPL has also been studied using animal models of APS. Blockade of its activation by neutralizing antibodies prevented the clots formation and endothelial cell activation and aPL failed to exert their pro-coagulant effect in C3 and C5 deficient mice (78, 79).

Patients with catastrophic APS or multiple arterial thrombosis refractory to standard therapy have benefited from the use of medications such as eculizumab (80–82). However, chronic administration of this treatment to prevent thrombotic

events would have a high cost, so it would be restricted to situations in which the development of coagulation is more probable. In this respect, the evaluation of B2-CIC could be a biomarker to assess the disease activity in APS patients, indicating those with a higher complement consumption who could benefit from the administration of this therapy. Further studies are needed to evaluate this possible hypothesis and to confirm the association with complement consumption in patients with thrombotic events.

A predominant presence of B2M-CIC (32.3%) has been observed as regards the prevalence of B2G-CIC (11.6%). This lower presence of B2G-CIC could be due to a more efficient IgG clearance-system. B2-CIC containing IgG or IgM have the capacity to activate complement, with the deposition of C3b and iC3b on these and making them accessible for the clearance in the liver and spleen by phagocytosis (through CR3 and CR4 receptors on macrophages) (83, 84). However, a lower clearance capacity of IgM-based B2-CIC through CR1 has been described compared to the removal of those formed by IgG (85). In addition, B2G-CIC can also be eliminated by the Fcγ receptors system present on immune cells (86). This additional mechanism is not present for B2-CIC formed by the IgM isotype. Only one receptor has been described for Fcμ of IgM and this receptor is involved in the regulation of immune response but does not perform clearance functions (87, 88). It may be possible that B2-CIC could only be detected in those patients when their clearance system is not sufficient enough to eliminate all of the B2-CIC because more quantity than normal has been generated.

The main limitation of our study is the absence of a control group made up of asymptomatic individuals with aPL (that is, individuals with aPL but with no history of APS clinical events and no concurrence of non-criteria symptoms associated with the syndrome). This group would be difficult to obtain because the evaluation of a large number of subjects would be necessary in order to recruit an adequate sample size of aPL-positive individuals without clinical manifestations and this would require extensive material and human investments. This is a retrospective study and in most of the cases the samples were not collected at the acute stage of the disease but rather during the follow-up. Since no longitudinal samples were included, it was not possible to determine whether the presence of B2-CIC increases at the time of the acute event and would thus constitute a risk factor for the development of APS events. In addition, it is difficult to find an association between the presence of B2-CIC and severe disease events due to the study design. All patients had a history of an APS event and 91% of them were taking preventive anticoagulant therapy, so the assessment of potential recurrences of severe clinical manifestations would be challenging. This work is fundamentally an epidemiological study that aims to find clinical and analytical associations with the presence of B2-CIC. For these reasons, further studies are needed to describe and confirm whether the observed thrombocytopenia and decreased C3 and C4 levels are caused by the binding of B2-CIC to the platelet surface and complement, respectively. Immunochemical studies should also be performed to confirm that immune-complexes integrating both B2GP1 and antibodies are capable of incorporating complement factors, which could initiate the activation of the classical pathway.

In conclusion, the presence of B2-CIC could contribute to higher complement and platelets consumption and therefore to a higher incidence of thrombocytopenia in patients with thrombotic APS.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Clinical Research Ethics Committee of the Hospital Universitario 12 de Octubre, Madrid, Spain. The patients/participants provided their written informed consent to participate in this study.

Author contributions

LN, LS, MS and AS conceived and designed the study. AD, LA, AT, MMaś, SS, MI, KK, MMan, FR, NS, MMil, JS, DR, IC, MR, MB, DP and YS contributed to the recruitment of patients, collection of samples and clinical data acquisition. LN and SG performed the B2-CIC quantification. LN, AS, and MS incorporated clinical and analytical information to the database, performed the first data analysis, wrote the first draft of the article and made all the changes suggested by the co-authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.957201/full#supplementary-material

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Frequency of positive antiphospholipid antibodies in pregnant women with SARS-CoV-2 infection and impact on pregnancy outcome: A single-center prospective study on 151 pregnancies

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Background: At the beginning of the SARS-CoV-2 pandemic, there was a lack of information about the infection's impact on pregnancy and capability to induce *de novo* autoantibodies. It soon became clear that thrombosis was a manifestation of COVID-19, therefore the possible contribution of *de novo* antiphospholipid antibodies (aPL) raised research interest. We aimed at screening SARS-CoV-2 positive pregnant patients for aPL.

Methods: The study included consecutive pregnant women who were hospitalized in our Obstetric Department between March 2020 and July 2021 for either a symptomatic SARS-CoV-2 infection or for other reasons (obstetric complications, labour, delivery) and found positive at the admission nasopharyngeal swab. All these women underwent the search for aPL by means of Lupus Anticoagulant (LA), IgG/IgM anti-cardiolipin (aCL), IgG/IgM anti-beta2glycoprotein I (aB2GPI). Data about comorbidities, obstetric and neonatal complications were collected.

Results: 151 women were included. Sixteen (11%) were positive for aPL, mostly at low titre. Pneumonia was diagnosed in 20 women (5 with positive aPL) and 5 required ICU admission (2 with positive aPL). Obstetric complications occurred in 10/16 (63%) aPL positive and in 36/135 (27%) negative patients. The occurrence of HELLP syndrome and preeclampsia was significantly associated with positive aPL (p=0,004). One case of maternal thrombosis occurred in an aPL negative woman. aPL positivity was checked after at least

12 weeks in 7/16 women (44%): 3 had become negative; 2 were still positive (1 IgG aB2GPI + IgG aCL; 1 IgM aB2GPI); 1 remained positive for IgG aCL but became negative for aB2GPI; 1 became negative for LA but displayed a new positivity for IgG aCL at high titre.

Conclusions: The frequency of positive aPL in pregnant women with SARS-CoV-2 infection was low in our cohort and similar to the one described in the general obstetric population. aPL mostly presented as single positive, low titre, transient antibodies. The rate of obstetric complications was higher in aPL positive women as compared to negative ones, particularly hypertensive disorders. Causality cannot be excluded; however, other risk factors, including a full-blown picture of COVID-19, may have elicited the pathogenic potential of aPL and contributed themselves to the development of complications.

KEYWORDS

anti-phospholipid antibodies, anti-phospholipid syndrome, anti-beta2glycoprotein I antibodies, COVID-19, SARS-CoV-2, pregnancy morbidity, HELLP syndrome, preeclampsia

Introduction

The SARS-CoV-2 pandemic has been affecting healthcare systems worldwide since 2020. At the beginning, there was a lack of information about the impact of the infection on pregnancy and its capability to induce *de novo* autoantibodies. It soon became clear that thrombosis was a manifestation of COVID-19, therefore the possible contribution of *de novo* antiphospholipid antibodies (aPL) raised research interest, as these autoantibodies are directly involved in the pathogenesis of vascular and obstetric complications (1), leading to the so-called picture of antiphospholipid syndrome (APS).

Current data suggest that pregnant people with SARS-CoV-2 symptomatic infection have increased risk of caesarean section, preterm birth and fetal distress, neonatal complications, isolated hypertension, preeclampsia/eclampsia, HELLP syndrome (characterized by haemolysis, elevated liver enzyme levels, low platelets counts) (2), intensive care unit (ICU) admission and death compared to women without COVID-19 during pregnancy (3, 4). Some of the most frequent maternal complications of COVID-19, like preeclampsia and gestational hypertension, may be also related to autoimmune diseases, in particular to the presence of aPL. Obstetric APS is characterized by fetal loss, recurrent early miscarriages, intrauterine growth restriction (IUGR), severe preeclampsia or HELLP syndrome (5, 6).

Therefore, the primary objective of this study was to evaluate the presence of antiphospholipid antibodies in pregnant patients with SARS-CoV-2 infection who were admitted to our hospital. The secondary objective was to understand whether the association between aPL positivity and COVID-19 could increase the risk of obstetric and neonatal complications.

Materials and methods

This is a single-center prospective study on consecutive SARS-CoV-2 positive pregnant women who were hospitalized to the Obstetric Department of the ASST Spedali Civili of Brescia between March 2020 and July 2021. These women were admitted either for symptomatic SARS-CoV-2 infection or for other reasons (i.e. obstetric complications, labour and delivery) and found positive at the admission nasopharyngeal swab. The study was performed according to the principles of the Declaration of Helsinki and was approved by the Local Ethics Committee (approval number NP4187). All patients gave their written informed consent.

All these women underwent search for aPL by means of Lupus Anticoagulant (LA), IgG/IgM anti-cardiolipin (aCL), IgG/IgM anti-beta2glycoprotein I (aB2GPI). IgG/IgM aCL and aB2GPI were detected by chemiluminescence immunoassay (Bioflash ®, INOVA Diagnostics, Werfen Group, Barcelona, Spain); LA test was performed according to the ISTH guidelines (7), using diluted Russel Viper Venom Time (dRVVT) and Silica Clotting Time (SCT) as screening tests (Werfen Group, Barcelona, Spain). Patients who tested positive for aPL were asked to recheck the tests after at least 12 weeks.

Data about symptoms related to COVID-19 were collected: dyspnea, pneumonia (clinically and radiologically documented by chest CT), fever, cough and pharyngodynia, dysgeusia or ageusia, diarrhea or vomiting. Patients with one or more of these symptoms were classified as symptomatic.

Information in pregnancy outcomes was collected: postpartum hemorrhage (PPH) (defined as losses greater than 500 ml for vaginal delivery and 1000 ml for cesarean section), need for blood transfusion, borderline blood pressure values (systolic blood pressure –SBP- between 130 and 140 mmHg and/or diastolic blood pressure –DBP- between 80 and 90 mmHg), hypertension (SBP greater than 140mmHg and/or DBP greater than 90mmHg), threatened preterm delivery (prior to 37 weeks of pregnancy), HELLP syndrome, FGR (fetal growth restriction, refers to fetus who does not achieve the expected *in utero* growth potential due to genetic or environmental factors) (8, 9), miscarriage, preterm premature rupture of membranes (pPROM), oligohydramnios (maximum vertical pocket <2 cm), thromboembolic events, preeclampsia (gestational hypertension accompanied by one or more of the following new-onset conditions at or after 20 weeks' gestation: proteinuria or other maternal organ dysfunction like acute kidney injury, liver involvement, neurological complications, haematological complications as thrombocytopenia, uteroplacental dysfunction or stillbirth) (10), abruptio placentae (ultrasound or clinically defined), and ICU admission. Regarding newborns, we collected data on sex, gestational age, birth weight, SGA (small for gestational age; below the 10^{th} percentile of body weight), LGA (large for gestation age; above the 90^{th} percentile of body weight) (11) and the presence of any neonatal complications.

The population was divided in two groups: "antiphospholipid negative" and "antiphospholipid positive". The latter was further divided into three subgroups according to aPL profile: "single positive", "double positive" and "triple positive" (12).

Statistical analysis

The database was formatted through the Microsoft-Excel® software and later imported from the IBM-SPSS® software ver. 28.0.1 (IBM SPSS Inc. Chicago, Illinois). The use of the Stata® software ver. 17.0 (Stata Corporation, College Station, Texas) was also considered for comparisons or implementations of test output. Normality of the distributions was assessed using the Kolmogorov-Smirnov test. Categorical variables were presented as frequencies or percentages and compared with the use of the Chi-Square test and the Fisher's exact test, as appropriate; associations of the crosstabs were verified using standardized adjusted residuals; when the matrix was 2x2 the odds ratios were calculated. Continuous variables were presented as means (in case of a normal distribution), or medians, IQR and interval (in case of a skewed distribution).

Logistic regressions were also used to calculate the associations between predictors covariates and each of the outcome variables Y. Then, the association of the dependent variables which showed significance to univariate regression on predictors covariates (one-by-one) was tested by IBM SPSS Neural Network analysis following the Multilayer Perceptron (MLP) procedure while performing a sensitivity analysis in order to compute the importance of each predictor in determining the neural network. A two-sided α level of 0.05 was considered significant for all tests.

The authors had full access to and take full responsibility for the integrity of the data.

Results

During the enrollment period, 151 women were tested for aPL: 87 (58%) during pregnancy and 64 (42%) in the immediate postpartum period. Their mean age was 33,3 years (IQR 29,3 – 37,5) with a median gestational age at delivery of 39 weeks (IQR 32,4 – 40).

Sixteen patients (11%) tested positive for at least one assay for aPL: 12/16 (75%) were single positive and 4/16 (25%) were double positive, all of them with positive IgG and/or IgM aB2GPI. No patient had a triple positive aPL profile. Nine patients presented a LA positive test; six were positive for aCL IgG and/or IgM; five were positive for aB2GPI IgG and/or IgM. Table 1 shows the comparison between aPL positive and aPL negative groups regarding: presence of COVID-19 symptoms and related treatment, presence of comorbidities, obstetric complications and neonatal complications.

Forty (26%) women presented with symptoms of COVID-19 at the time of admission, the mean age was 35,4 years (IQR 32,6 –39,5) with a median gestational age at delivery of 30,6 weeks (IQR 24,8 – 33,7). 6/16 (36%) aPL positive patients displayed a SARS-CoV-2 symptomatic infection: 5 with pneumonia, of which 2 required Intensive Care Unit (ICU) admission, and 1 with dyspnea. In the aPL negative group 34/135 (25%) had COVID-19 symptoms: 15 with pneumonia, of which 3 required ICU admission, and the remaining with transient dyspnea and minor symptoms (fever, cough, etc.). 111/151 (74%) were asymptomatic for SARS-CoV-2: the mean age was 32,6 years (IQR 28,8 – 36,9) with a median gestational age at delivery of 39,4 weeks (IQR 38,7 – 40,2).

41/151 (27%) women had one or more comorbidities. Nine patients with comorbidities were in the aPL positive group: autoimmune disorders (2 ANAs positivity), chronic metabolic disorders (2 hypothyroidism, 1 obesity), chronic infectious disorder (1 chronic hepatitis B), obstetric disorders (2 multiple abortions) and other comorbidities (2 behavioural disorders, 1 coagulation factor XII deficiency, 1 hypoacusis, 1 metachromatic leukodystrophy). The remaining 32 women with comorbidities were in the aPL negative group: autoimmune disorders (2 Crohn's disease, 2 diabetes mellitus, 1 ANAs positivity, 1 anti-

TABLE 1 Characteristics of 151 patients with SARS-CoV-2 divided upon the positivity for antiphospholipid antibodies (aPL).

		Total patients (n=151)	aPL negative (n=135)	aPL positive (n=16)	Single Positive (n=12)	Double Positive (n=4)	LA + (n=9)	aCL + (n=6)	aB2GPI+ (n=5)
SARS-CoV-2 symptomatic infection		40/151 (26%)	34/135 (25%)	6/16 (36%)	5/12 (42%)	1/4 (25%)	4/9 (44%)	2/6 (33%)	1/5 (20%)
Treatment	Antiviral drugs	13/151 (9%)	9/135 (7%)	4/16 (25%)	3/12 (25%)	1/4 (25%)	3/9 (33%)	1/6 (17%)	1/5 (20%)
	Corticosteroids	21/151 (14%)	16/135 (12%)	5/16 (31%)	4/12 (3%)	1/4 (25%)	3/9 (33%)	2/6 (33%)	1/5 (20%)
	Low dose aspirin	2/151 (1%)	2/135 (1%)	0	0	0	0	0	0
	LMWH	55/151 (36%)	47/135 (35%)	8/16 (50%)	6/12 (50%)	2/4 (50%)	4/9 (44%)	3/6 (50%)	3/5 (60%)
	HCQ	2/151 (81%)	0	2/16 (12%)	2/12 (17%)	0	1/9 (11%)	1/6 (17%)	0
ICU Admission		5/151 (3%)	3/135 (2%)	2/16 (12%)	1/12 (8%)	1/4 (25%)	1/9 (11%)	1/6 (17%)	1/5 (20%)
Comorbidities		41/151 (27%)	32/135 (24%)	9/16 (56%)	7/12 (58%)	2/4 (50%)	6/9 (67%)	2/6 (33%)	3/5 (60%)
Obstetric Complications		46/151 (31%)	36/135 (27%)	10/16 (62%)	6/12 (50%)	4/4 (100%)	6/9 (67%)	3/6 (50%)	5/5 (100%)
Neonatal Complications		19/151 (13%)	16/135 (12%)	3/16 (19%)	1/12 (8%)	2/4 (50%)	1/9 (11%)	2/6 (33%)	2/5 (40%)

aPL, antiphospholipid antibodies; LA, lupus anticoagulant; aCL, anti-cardiolipin; aB2GPI, anti-beta2glycoprotein I; ICU, Intensive care unit; LMWH, Low Molecular Weight Heparin; HCQ, Hydroxychloroquine.

thyreoglobulin antibodies positivity, 1 myasthenia gravis, 1 systemic sclerosis, 1 ulcerative rectocolitis), chronic metabolic disorders (10 hypothyroidism, 4 obesity, 1 hyperparathyroidism, 1 hypovitaminosis D), neoplastic disorders (1 anamnestic ovarian cancer, 1 anamnestic papillary thyroid carcinoma), chronic infectious disorder (1 HIV infection), obstetric disorders (1 endometriosis) and other comorbidities (4 asthma, 3 chronic hypertension, 1 anemia, 1 cholecystitis, 1 cholelithiasis, 1 chronic renal failure, 1 factor V Leiden mutation, 2 hemoglobinopathies, 1 lower extremities chronic venous disease, 1 splenectomy, 1 proteinuria, 1 rheumatic mitral regurgitation).

The obstetric complications that occurred in the whole cohort were: 11 postpartum hemorrhage, 11 preeclampsia, 5 isolated hypertension, 5 IUGR, 5 SGA, 3 chorioamnionitis, 3 hepatic disease, 3 oligohydramnios, 3 urinary tract infections, 3 threat of preterm delivery, 2 abnormal cardiotocography, 2 twin pregnancy, 1 abruptio placentae, 1 cervical dystocia, 1 disseminated intravascular coagulation, 1 gestational diabetes, 1 HELLP syndrome, 1 hyperemesis, 1 iatrogenic bladder injury, 1 internal abortion, 1 LGA, 1 maternal-fetal transfusion, 1 multiple placental infarctions, 1 placenta previa major, 1 pPROM, 1 proteinuria, 1 sepsis, 1 twin pregnancy with twin-twin transfusion syndrome.

In particular, we observed 11 cases of preeclampsia, of which 4 in aPL positive patients (1 in a patient with isolated LA positivity, and 3 in the double positivity group,2 with IgG aCL and IgG aB2GPI, 1 with LA and IgG aB2GPI); 12 cases of postpartum hemorrhage, of which one occurred in a patient with isolated positive IgM aB2GPI and one in a patient with IgG aCL and IgG aB2GPI; one case of HELLP syndrome in a double positive patient (IgG aCL and IgG aB2GPI). The latter was a 39-years-old primigravida with asymptomatic SARS-CoV-2 infection who was hospitalized for an elective cesarean section. The medical history reported behavioral disorders on treatment with sertraline, trazodone, alprazolam and amisulpride. The delivery was complicated by the onset of preeclampsia, HELLP syndrome, and postpartum hemorrhage; there were no neonatal complications. After 12 weeks, aPL tests were repeated and IgG aCL remained positive while IgG aB2GPI became negative.

One or more neonatal complications occurred in 19/151 (13%) cases. Three in the aPL positive group: 2 respiratory distress syndromes, 1 admission to neonatal ICU, 1 hypoglycemia and 1 neonatal necrotizing enterocolitis. The other neonatal complications observed in the aPL negative group were: 9 admissions to neonatal ICU, 8 respiratory distress syndrome, 6 hypoglycemia, 4 hyperbilirubinemia, 1 feto-maternal transfusion, 1 sepsis, 1 transient tachypnea of the newborn, 1 urinoma.

The statistical analysis about the obstetric complications associated with placental insufficiency due to aPL positivity was described in Table 2. Table 3 collected the statistically significant results of the univariate logistic regression of confounding factors analyzed (only the significant ones shown): comorbidities (in particular obesity and hypothyroidism), SARS-CoV2 symptomatic infection and related treatment. Focusing on the objective of the study, the strongest inferential non-parametric significant association was found between HELLP syndrome and/or preeclampsia and positive aPL (p value = 0,004). Regarding the 5 patients with positive IgG and/or IgM aB2GPI (isolated or with

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TABLE 2 Pregnancy complications occurring in 151 patients, divided upon profile for antiphospholipid antibodies (aPL).

	Negative aPL (n=135)	Positive aPL (n=16)	P-value	Single positive aPL (n=12)	P-value	Double positive aPL (n=4)	P-value	aB2GPI positive (n=5)	P-value
Abruptio placentae	1/135(1%)	0	NS	0	NS	0	NS	0	NS
Gestational hypertension	5/135 (4%)	0	NS	0	NS	0	NS	0	NS
HELLP syndrome	0	1/16 (6%)	0,004	0	NS	1/4 (25%)	<0,001	1/5 (20%)	<0,001
ICU admission	3/135 (2%)	2/16 (13%)	0,030	1/12 (8%)	NS	1/4 (25%)	0,038	1/5 (20%)	0,034
IUGR	4/135 (3%)	1/16 (6%)	NS	0	NS	1/4 (25%)	NS	1/5 (20%)	0,034
LGA	0	1/16 (6%)	0,04	1/12 (8%)	NS	0	NS	0	NS
Maternal-fetal trasfusion	1/135 (1%)	0	NS	0	NS	0	NS	0	NS
Maternal thrombosis	1/135 (1%)	0	NS	0	NS	0	NS	0	NS
Neonatal complications	16/135 (12%)	3/16 (19%)	NS	1/12 (8%)	NS	2/4 (50%)	NS	0	NS
Oligohydramnios	3/135(2%)	0	NS	0	NS	0	NS	0	NS
PPH	10/135 (7%)	2/16 (13%)	NS	1/12 (8%)	NS	1/4 (25%)	NS	2/5 (40%)	0,007
Preeclampsia	7/135 (5%)	4/16 (25%)	0,004	1/12 (8%)	NS	3/4 (75%)	<0,001	3/5 (60%)	<0,001
pPROM	0	1/16 (6%)	0,004	1/12 (8%)	NS	0	NS	0	NS
SGA	4/135 (3%)	1/16 (6%)	NS	1/12 (8%)	NS	0	NS	1/5 (20%)	0,034
Threatened preterm delivery	2/135 (1%)	1/16 (6%)	NS	0	NS	1/4 (25%)	NS	1/5 (20%)	NS

aB2GPI, anti-beta2glycoprotein I; aPL, antiphospholipid antibodies; HELLP, hemolysis, elevated liver enzymes, and low platelets; ICU, Intensive care unit; IUGR, Intrauterine growth restriction; LGA, large for gestational age; NS, not significant; PPH, post-partum hemorrage; pPROM, preterm premature rupture of membranes; SGA, small for gestational age.

positive aCL or LA) a statistically significant association with HELLP syndrome and/or preeclampsia (p value < 0,001) was confirmed. The multivariate logistic regression analysis did not confirm these associations; however, using the MLP procedure, we could verify the dependence of both preeclampsia and HELLP syndrome mainly on aB2GPI and aPL positivity (Figure 1).

Among the 16 women who tested positive for aPL, 7 (44%) had their aPL re-checked after at least 12 weeks: 3 became negative; 2 remained stable for both type and titre; 1 previously double positive patient turned out as a single positive; 1 single positive patient had a switch from positive LA to positive IgG aCL. Table 4 shows descriptive data for each of the 16 aPL positive women.

Discussion

To the best of our knowledge, this is the first prospective study to investigate SARS-CoV-2 infection as a possible trigger of aPL positivity in pregnant women, with the aim of evaluating whether the overlap of these two conditions may result in an increased rate of obstetric and neonatal complications.

It is well recognized that infectious agents can play a dual role in the etiopathogenesis of APS, by acting as the initial trigger of the production of antibodies cross-reacting with aB2GPI and infectious peptides, and by inducing an inflammatory response. This is the so-called "two-hits theory" in which pathogenic aB2GPI act as the first hit and inflammatory response as the second one (13, 14). Moreover, several studies have shown that aCL occurred frequently during viral infections, particularly in HIV (49,7%), hepatitis B virus (HBV) (24%), and hepatitis C virus (HCV) (20%), while anti-B2GPI very rarely detected; aPL during the course of infections did not generally associate with thrombosis or other manifestations of APS (15).

The link between coagulopathy due to COVID-19 and positive aPL has been addressed in several studies, trying to define aPL as either an "epiphenomenon" or an active player in the pathogenesis (16). To shed light on this issue, Borghi et al. investigated the fine specificity of aPL detected in patients who were hospitalized for COVID-19-related thrombosis (17). A small group of patients (12,3%) were found to be positive for aPL at medium/low titre using chemiluminescence immunoassay, namely IgG/IgM/IgA aCL in 9,8/6,6/2,5% of patients and/or IgG/IgA/IgM aB2GPI in 5/5/0,8%. These aPL had different features from those found in patients with definite APS (reactivity against domain 1 and domain 4/5 of B2GPI was found in nearly 5% of the aPL positive cases) and there was no association between aPL positivity and thrombotic events. These aPL were mostly of the IgM isotype and at low titre, which are features classified under the definition of "low risk" aPL profile

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TABLE 3 Univariate logistic regression of statistically significant parameters and variables involved in pregnancy complications.

		aPL positivity	B2GPI positivity	Comorbidities	Obesity	Hypothyroidism	SARS-CoV2	SARS-CoV2 treatment
Preeclampsia	P-value	0,004	<0,001	0,005	–	–	–	0,003
	OR (95% CI)	6,1 (1,6-2,3)	25,9 (3,8-177,6)	5,5 (1,5-19,8)	–	–	–	8,1
Abruptio placentae	P-value	–	–	–	<0,001	–	–	(1,7-39,0)
	OR (95% CI)	–	–	–	1,2 (0,8-1,9)	–	–	–
HELLP Syndrome	P-value	0,004	<0,001	–	–	–	–	–
	OR (95% CI)	1,1 (0,9-1,2)	1,2 (0,8-1,9)	–	–	–	–	–
ICU admission	P-value	0,030	0,034	–	0,034	–	<0,001	–
	OR (95% CI)	6,3 (1,0-40,9)	8,9 (0,8-98,4)	–	8,9 (0,8-98,4)	–	1,1 (1,0-1,3)	0,005
IUGR	P-value	–	0,034	–	–	–	–	1,1
	OR (95% CI)	–	0,9 (0,8-98,4)	–	–	–	–	(1,0-1,2)
LGA	P-value	0,004	–	–	<0,001	–	–	0,005
	OR (95% CI)	1,1 (0,9-1,2)	–	–	1,3 (0,8-1,9)	–	–	1,1
SGA	P-value	–	0,034	–	–	–	–	(1,0-1,2)
	OR (95% CI)	–	8,9 (0,8-98,4)	–	–	–	–	–
PPH	P-value	–	0,007	–	–	–	–	–
	OR (95% CI)	–	9,1 (1,4-60,7)	–	–	–	–	–
pPROM	P-value	0,004	–	–	–	0,001	–	–
	OR (95% CI)	1,1 (0,9-1,2)	–	–	–	1,1 (0,9-1,3)	–	–
Threat of preterm delivery	P-value	–	0,003	–	–	–	–	–
	OR (95% CI)	–	18 (1,3-241,8)	–	–	–	–	–

aB2GPI, anti-beta2glycoprotein I; aPL, antiphospholipid antibodies; CI, confidence intervals; HELLP, hemolysis, elevated liver enzymes, and low platelets; ICU, Intensive care unit; IUGR, Intrauterine growth restriction; LGA, large for gestational age; OR, odds ratio; PPH, post-partum hemorrhage; pPROM, preterm premature rupture of membranes; SGA, small for gestational age.

(12). Although this aPL profile is not strongly predictive of vascular events in APS, it is important to keep in mind that COVID-19 patients suffer from an acute form of systemic inflammation with complement activation (18), which may be responsible for endothelial perturbation. In this context, since aB2GPI can accumulate on the activated endothelium at high density, even low titers of aPL may become a pathogenic trigger for thrombosis. While transitory aPL are likely to be clinically non-significant in COVID-19 patients as in other infections (19), they may play a role in risk stratification of selected patients, with aPL being an additional risk factor acting synergistically with others. In this regard, we underline that only one case of maternal thrombosis was observed in our study, and it occurred in a patient with COVID-19 and negative aPL, confirming that COVID-19 itself can favor clots during a thrombophilic state like pregnancy.

Turning to obstetric APS, an aPL profile which is considered at “low risk” for thrombosis can actually mediate substantial

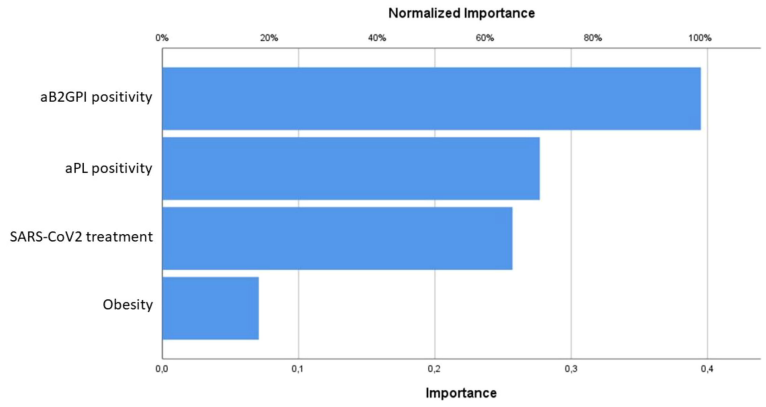

FIGURE 1
Predictive models resulting from the Multilayer Perceptron procedure for preeclampsia and HELLP syndrome, mainly influenced by aB2GPI and aPL positivity.

damage, as high levels of B2GPI can be found in the placenta and bind aB2GPI antibodies, even at low titre (20). The presence of aPL was indeed found to be associated with an increased rate of obstetrical complications and fetal loss in the general obstetric population (21–24). In particular, it was demonstrated that the pathogenic mechanisms responsible for preeclampsia could be linked to a damage of the endothelium, ultimately leading to the release of vasoactive compounds (25) and that aB2GPI antibodies are able to promote endothelial activation (26). Therefore, Faden et al. assumed that the endothelial damage promoted by aB2GPI antibodies could be the starting point for the cascade of events leading to the preeclamptic syndrome and HELLP syndrome (27).

In November 2021, the US Centers for Disease Control and Prevention confirmed that pregnant women who develop COVID-19 have an increased risk of stillbirth, especially with the Delta type variant. Studies have also shown that placentas found positive for SARS-CoV-2 are typically characterized by a wide spectrum of pathological findings such as villous trophoblast necrosis, chronic histiocytic intervillositis and increased fibrin up to the level of massive perivillous massive deposition. These pathological lesions, in some cases together with placental hemorrhage, thrombohematomas and villitis, result in severe and diffuse placental parenchymal destruction (28). Before the pandemic, these lesions and subsequent placental insufficiency were typically associated with malperfusion conditions resulting from maternal diseases (maternal hypertension, diabetes mellitus, coagulopathies), fetal diseases (umbilical cord problems, abnormalities of placental development or placental implantation), environmental exposure to cigarette smoke, abruptio placentae, etc. Among the coagulopathies of autoimmune origin that are most associated with obstetric and neonatal complications there is indeed APS. Therefore, the pathogenic mechanisms underlying placental dysfunction and obstetric complications are complex, making it difficult to attribute adverse events to the direct viral effect, to the presence of aPL, or the combination of both factors.

Our study suggested that SARS-CoV-2 infection during pregnancy was not associated with the development of aPL. Recent studies reported the estimated prevalence of aPL positivity in the general population between 5 and 10%, in agreement with what we found in our patient cohort (11%) (24, 29–32). Moreover, we found a frequency of aB2GPI positivity similar to that described in a pioneer study that was conducted in our hospital by enrolling hundreds of pregnant women from the general obstetric population; we can assume that these figures from decades ago are still valid as the methods used for aPL detection have been validated for reproducibility overtime (27). Additionally, if we consider the total number of patients who presented with at least one obstetric complication, the frequency of positive aPL (28%) in this subgroup is similar to the one described in negative SARS-CoV-2 obstetric population (32–34).

In our cohort, obstetric complications occurred more frequently in aPL positive patients than in aPL negative ones. We might speculate that the interplay between aPL and SARS-CoV-2 infection could increase the risk of obstetric complications. By restricting the analysis to patients with positive aB2GPI, we found a significant association with complications mediated by placental dysfunction, namely preeclampsia and HELLP syndrome.

However, our study has limitations. We were not able to collect a control group of pregnant women with a negative swab test for SARS-CoV-2, due to the burden of multiple and severe wves of COVID-19 that hit our area. Moreover, the small number of aPL positive women and possible aPL-related

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TABLE 4 Description of the 16 patients who resulted positive for antiphospholipid antibodies (aPL).

	Age	Gestational age	Reason for hospitalization	Comorbidities	1st aPL test	Pneumonia	Obstetric complications	Neonatal complications	2nd aPL test
1	35y	39+2	Iterative caesarean section and tubal sterilization	ANA positivity	aB2GPI IgM+	No	Small for gestational age; Post-partum hemorrhage	No	aB2GPI IgM +
2	27y	41+3	Induction of labor	No	aCL IgG +	No	No	No	ND
3	28y	41+5	Induction of labor	No	LA +	No	No	No	ND
4	33y	10+4	SARS-CoV-2 bilateral interstitial pneumonia	Hypothyroidism	aCL IgG +	Yes	No	No	Negative
5	39y	38+3	Caesarean section	Behavioural disorders	aCL IgG + aB2GPI IgG+	No	Post-partum haemorrhage; Preeclampsia; HELLP syndrome	No	aCL IgG+ aB2GPI IgG -
6	28y	34+5	Suspected preeclampsia	No	aCL IgG + aB2GPI IgG+	No	Preeclampsia; Intrauterine growth restriction	Hypoglycaemia; Neonatal Intensive Care Unit admission	ND
7	27y	23+3	Urosepsis	Hypoacusis; Deficit XII factor	LA+ aB2GPI IgG+	No	Preeclampsia	No	Negative
8	19y	31	SARS-CoV-2 bilateral interstitial pneumonia	No	aCL IgG+ aB2GPI IgG+	Yes	Threat of preterm delivery	Respiratory distress syndrome	aCL IgG+ aB2GPI IgG +
9	35y	33	SARS-CoV-2 bilateral interstitial pneumonia; Caesarean section	ANA positivity; Hepatitis B	LA +	Yes	Preeclampsia	Respiratory distress syndrome; Neonatal necrotizing enterocolitis	ND
10	34y	40+6	Labour	No	aCL IgG+	No	No	No	ND
11	35y	13+6	Internal abortion	Multiple abortions	LA +	No	Internal abortion	No	ND
12	39y	18+3	SARS-CoV-2 bilateral interstitial pneumonia	Hypothyroidism, Multiple abortion	LA +	No	No	No	Negative
13	38y	25+5	Premature rupture of membranes	Hypothyroidism	LA +	No	Premature rupture of membranes	No	LA – aCL IgG + at high titre
14	28y	10+5	Hyperthyroidism	No	LA +	No	Hyperemesis	No	ND
15	32y	38+6	SARS-CoV-2 bilateral interstitial pneumonia	Behavioural disorders; Metachromatic leukodystrophy; Obesity	LA +	Yes	Urinary tract infection; Large for gestational age; Multiple placental infarctions	No	ND
16	40y	–	SARS-CoV-2 bilateral interstitial pneumonia	No	LA +	Yes	No	No	ND

aB2GPI, anti-beta2glycoprotein I; aCL, anti-cardiolipin; aPL, antiphospholipid antibodies; HELLP, hemolysis, elevated liver enzymes, and low platelets; LA, lupus anticoagulant; ND, No Data.

manifestations limited the validity of statistical analysis. The heterogeneity of the cohort in terms of comorbidities, reasons for hospitalization, drugs intake, and gestational age also limited the interpretation of findings. For instance, the woman with confirmed positive aPL who developed HELLP syndrome had been taking antipsychotic medications, which are known to increase the chances for positive aPL (35).

In conclusion, our study showed that the frequency of positive aPL in pregnant women with SARS-CoV-2 infection is not greater than that described in the general obstetric population. Therefore, the infection itself does not seem to elicit an autoimmune response towards phospholipid-binding proteins like B2GPI. It might be possible that some women already carried aPL prior to the infection and the interaction

between these two factors can facilitate the occurrence of obstetric complications, with particular reference to placental dysfunction leading to preeclampsia and HELLP syndrome. Based on our findings, aPL testing does not seem to be warranted in pregnant women with SARS-CoV-2 infection; however, aPL may contribute to risk stratification in women who present with concomitant risk factors and/or severe forms of COVID-19.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Brescia (approval number NP4187). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

LA, AT, FF, SZ, FP contributed to conception and design of the study. BN, MM, CZ, RO, SZ, FP, ES were involved in the enrollment of patients. MM, BN, GIG, EP, MGL, SP organized the database and performed the data collection. GIG, EP, CT performed the statistical analysis and prepared the tables. LA, GIG, EP wrote sections of the manuscript. All authors reviewed the manuscript and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Non-criteria antiphospholipid antibodies in antiphospholipid syndrome: Diagnostic value added

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Objective: Non-criteria antiphospholipid antibodies (aPLs) increase the diagnostic value for antiphospholipid syndrome (APS) and contribute to better recognition of seronegative APS (SNAPS). However, the clinical utility and the diagnostic value of non-criteria aPLs are inconsistent. This study aimed to investigate the prevalence and clinical significance of 7 non-criteria aPLs in a large APS cohort.

Methods: Seven non-criteria aPLs, including anti-phosphatidylserine/prothrombin (aPS/PT) antibodies IgG/IgA/IgM, anti-phosphatidylethanolamine antibodies (aPE) IgG/IgA/IgM, anti-Annexin V antibodies (aAnnexinV) IgG/IgA/IgM, anti-phosphatidylserine antibodies (aPS) IgM, aPS IgG, antibodies directed against a mixture of phospholipids (APhL) IgG, and APhL IgM were tested among 175 patients with APS, 122 patients with other autoimmune diseases (as disease controls), and 50 healthy controls.

Results: In the present study, the highest prevalence of non-criteria aPLs was seen in aAnnexinV (58.86%). APhL IgG and aPS IgM showed the highest specificity (95.35%) and aPS/PT showed the highest Youden index (0.3991) for the diagnostic value of APS. The aAnnexinV also showed the highest prevalence in SNAPS (43.3%), followed by APhL IgM (21.7%), aPE (16.7%) and aPS/PT (16.7%). APhL IgG, aPS/PT, and aPS IgG showed positive association with thrombotic events in APS patients [APhL IgG: odds ratio (OR) = 2.26, 95% confidence interval (CI) 1.18-4.34, p = 0.013; aPS/PT: OR = 2.48, 95% CI: 1.32-4.69, p = 0.004; aPS IgG: OR = 1.90, 95% CI 1.01-3.60, p = 0.046; respectively). The inclusion of the non-criteria aPLs increased the accuracy of APS diagnosis from 65.7% to 87.4%.

Conclusion: Our data provide evidence that adding the non-criteria aPLs can improve the diagnostic accuracy in APS. APhL IgG, aPS/PT, and aPS IgG may be potential biomarkers to predict the risk of thrombosis in APS.

KEYWORDS

antiphospholipid syndrome, non-criteria antiphospholipid antibodies, thrombosis, APhL, aPS/PT antibody

Introduction

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder characterized by arterial and venous thrombosis and/or pregnancy morbidity with the presence of persistent antiphospholipid antibodies (aPLs) (1). According to the 2006 Sydney Classification criteria for definite APS (2), the IgG/IgM anticardiolipin antibodies (aCL), anti-β2-glycoprotein I antibodies (aβ2GPI), and lupus anticoagulant (LA) were defined as criteria aPLs. The three criteria aPLs are not only critical components in APS classification, but are also considered risk factors for thrombosis or pregnancy morbidity in APS (3, 4). They are also associated with APS "non-criteria" manifestations (5). All three criteria aPLs were included in two widely accepted risk score systems, i.e., APL-S (6) and the Global APS score (7). However, some patients exhibit clinical manifestations highly suggestive for the diagnosis of APS but persistently negative for criteria aPLs. These patients are defined as seronegative APS (SNAPS) (8).

To date, several non-criteria aPLs have been investigated to identify SNAPS better. The autoantigens specificity of these non-criteria aPLs includes different phospholipids, phospholipid binding proteins, and coagulation factors (9, 10). There are more than 30 known non-criteria aPLs in APS (11, 12). Among these, anti-phosphatidylserine/prothrombin antibodies (aPS/PT), aβ2GPI Domain I, IgA of aβ2GPI and aCL were highly specific for the identification of APS patients and have been the subject of previous investigations (9, 13–17). Of the non-criteria aPLs, aPS/PT are also included in the GAPSS and APL-S for risk stratification in APS patients (6, 7). Thus, the aPS/PT and aβ2GPI Domain I have been regarded as "first-line" non-criteria aPLs (18). However, the clinical significance of other non-criteria aPLs have not yet been investigated. These aPLs are still controversial because most existing studies evaluated only one or just a few non-criteria aPLs using different diagnostic assays, and have different study designs.

To better understand clinical significance of the non-criteria aPLs in APS, we evaluated the diagnostic value of seven non-criteria aPLs and their association with APS subphenotypes in a large APS cohort.

Methods

Patients

Consecutive patients who had APS ICD-9 code and were admitted to the Department of Rheumatology and Immunology, Peking University People's Hospital (PKUPH), were enrolled retrospectively in this study. The inclusion criteria were: 1) Patients fulfilled the 2006 Sydney criteria (2) (seropositive APS, SPAPS) or fulfilled the Sydney clinical criteria but were persistently negative for aCL, aβ2GPI, and LA at least on two separate occasions (seronegative APS, SNAPS) (8). At least one obstetric or one major non-obstetric or two minor non-obstetric "non-criteria" manifestations were also required for the classification of SNAPS (19). The obstetric, major, and minor non-obstetric "non-criteria" manifestations were shown in Supplementary Tables 1; 2) The serum of these patients were collected simultaneously and stored in -80°C freezer. The exclusion criteria were: 1) Patients with hereditary and other acquired thrombophilia disorders; 2) Incomplete medical records. At least two expert rheumatologists confirmed the diagnosis for patients.

The inclusion criteria of other autoimmune diseases were: 1) patients without thrombosis and pregnancy morbidity; 2) patients without APS. The patients with other autoimmune diseases were diagnosed according to current classification. The medical records were reviewed to obtain patients' demographic and clinical information. Demographic data, clinical data, co-morbidity and laboratory data were collected.

This study was approved by the ethics committees of Peking University People's Hospital (2019PHB253) and fulfilled the Declaration of Helsinki guidelines for the inclusion of humans in research.

Detection of criteria aPLs

IgG, IgM and IgA isotypes of aCL and aβ2GPI were detected using quantitative IMTEC ELISA kits (HUMAN Diagnostics, Inc, Wiesbaden, GER). According to the manufacturer, the cutoff values of positive aCL and aβ2 GPI were 45U/ml and 5U/ml, which were consistent with the ROC curve calculated by HC.

The lupus anticoagulant test was conducted as previously described (20). The simplified Dilute Russell's Viper Venom Test (dRVVT) was performed using the Stago STA Compact Hemostasis system. It used diluted activated partial thromboplastin time as screening tests by ISTH recommendations (21).

Detection of non-criteria aPLs

Antibodies against phosphatidylserine/prothrombin (aPS/PT) IgG/IgA/IgM were measured using quantitative ELISA kits (HUMAN Diagnostics, Inc, Wiesbaden, GER). The cut-off value of aPS/PT was 30U/ml, according to the manufacturer's instructions.

Anti-Annexin V antibodies (aAnnexinV) were detected using indirect solid-phase ELISA (HUMAN Diagnostics, Inc, Wiesbaden, GER) for the quantitative measurement of IgG, IgA, and IgM class autoantibodies against annexin V in human serum. Results above 25U/ml were considered positive.

Anti-phosphatidylethanolamine antibodies (aPE) IgG/IgA/IgM were measured using IMTEC ELISA kits with β2 GPI as a cofactor. According to the manufacturer's instructions, the cutoff value was ≥ 15U/ml.

Anti-phosphatidylserine (aPS) IgM and IgG were measured using quantitative IMTEC ELISA kits (HUMAN Diagnostics, Inc, Wiesbaden, GER) against phosphatidylserine/β2GPI. Results above 15U/ml were considered positive for both IgM and IgG.

Antigens of APhL were a mixture of negatively charged phospholipids. The APhL IgG and APhL IgM were measured using the APhL ELISA assay (Louisville APL Diagnostic, Inc, Louisville, KY, USA), and cutoff values of 15 GPL/MPL units were used as recommended by the manufacturer.

Statistical analysis

Variables with a normal distribution were presented as means with standard deviations or absolute numbers with percentages of the total. The data were presented as medians and interquartile ranges (IQRs) for variables with skewed distribution. The sensitivity, specificity, accuracy, Youden index, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), and negative likelihood ratio (NLR) were calculated for the diagnosis of APS. Logistic regression models were applied to assess the diagnostic values of different aPL combinations for APS. The receiver operating characteristic (ROC) curves were generated for single or combined aPLs, respectively. The area under the curve (AUC) were calculated to evaluate the diagnostic performance of the single or combined aPLs. Comparisons between non-criteria aPLs and clinical manifestations of APS were performed using the χ^2 test. Fisher's exact test was used if the expected number in a cell of a two-by-two table was less than five. Titers of non-criteria aPLs between groups were compared with the Mann-Whitney *U* test. The differences between groups were calculated by one-way ANOVA. Statistical significance was set at *p*-values less than 0.05. Statistical analysis was performed using SPSS v.15.0 (IBMCorp., Armonk, NY, USA) or R (version3.6.0).

Results

A total of 347 patients were included in this study. Among these patients, 175 APS patients were categorized into APS groups, 122 patients with other autoimmune diseases without thrombosis or obstetrical morbidity, and 50 healthy controls (HC) served as the control group.

There were 115 SPAPS patients, with 94 (81.7%) females. The mean age was 42.4 years. Among these patients, 80 (69.6%) patients had a history of thrombosis, 42 (44.7%) female patients had a history of pregnancy morbidity and 7 (7.4%) female patients had a history of both thrombosis and pregnancy morbidity (Table 1).

Sixty patients were included in the SNAPS group. In this SNAPS group, 55 (91.7%) were female patients with mean age of 38.2 years. Thrombosis was present in 25 (41.7%) patients, and pregnancy morbidity was present in 43 (78.2%) female patients. In addition, 8 (14.5%) patients had a history of thrombosis and pregnancy morbidity (Table 1). The rate of non-criteria clinical manifestation of APS was presented in Supplementary Table 2.

The disease control (DC) group included 42 patients with systemic lupus erythematosus (SLE), 26 patients with Sjögren's syndrome (SS), 17 patients with rheumatoid arthritis (RA), 17 patients with ankylosing spondylitis (AS), and 20 patients with osteoarthritis (OA). The baseline characteristics are presented in Table 1.

Prevalence and diagnostic values of aPLs

The aCL, aβ2GPI, and LA were present in 29 (25.2%), 62 (53.9%), and 96 (83.5%) of the SPAPS patients, respectively. The prevalence of the seven non-criteria aPLs were shown in Table 2. For the non-criteria aPLs, the presence of aAnnexinV, aPE, aPS/PT, aPS IgG, aPS IgM, APhL IgG, and APhL IgM in the SPAPS patients were 67.0%, 40.9%, 60.0%, 53.9%, 20.0%, 55.7%, and 19.1%, respectively, and were significantly higher than in the control groups.

The titers of these criteria and non-criteria aPLs among the different groups were illustrated in Supplementary Figure 1. No significant differences were observed in levels of aPS IgM and APhL IgM between SPAPS and SNAPS groups. Compared to the healthy controls, levels of all autoantibodies were significantly elevated in patients with SPAPS. Compared to the disease control groups, levels of aβ2GPI, aPS/PT, aPS IgG, and APhL IgG were increased dramatically in patients with SPAPS.

ACL exhibited the highest specificity of 99.42% but with a low sensitivity of 16.57%, followed by aβ2GPI (specificity of 98.26% and sensitivity of 34.29%). aAnnexinV exhibited the highest sensitivity of 58.86% but with the lowest specificity of 55.81%. LA displayed the highest Youden index of 0.5021 (specificity of 95.35% and sensitivity of 54.86%), followed by aPS/PT (Youden index of 0.3991)(Table 3). The diagnostic value for each APS subtypes were also analyzed. The Youden index of most criteria and non-criteria aPLs were higher in APS patients only with a history of thrombosis than in APS patients only with a history of pregnancy morbidity, and the same situation occurred between secondary and primary patients (Supplementary Tables 3–5).

To further evaluate the predictive value of these criteria and non-criteria aPLs for APS, the receiver operating characteristic (ROC) curves were plotted. Among these aPLs, aβ2GPI showed the most significant area under the curve (AUC = 0.746), followed by APhL IgG (AUC = 0.732) (Figure 1 and Supplementary Table 6).

TABLE 1 Demographic and clinical characteristics of the study population.

	SPAPS (N=115)	SNAPS (N=60)	OA (N=20)	RA (N=17)	SLE (N=42)	SS (N=26)	AS (N=17)	Healthy controls (N=50)
Mean age (years ± SD)	42.4 ± 15.3	38.2 ± 13.4	64.3 ± 12.6	60.3 ± 12.7	40.6 ± 15.1	58.2 ± 11.4	46.6 ± 16.1	42.4 ± 10.3
Sex (female), n (%)	94 (81.7)	55 (91.7)	17 (85.0)	16 (94.1)	40 (95.2)	26 (100.0)	7 (41.2)	33 (66.0)
Hypertension, n (%)	24 (20.9)	4 (6.7)	—	—	—	—	—	—
Diabetes mellitus, n (%)	11 (9.6)	2 (3.3)	—	—	—	—	—	—
Smoking status, n (%)	17 (14.8)	4 (6.7)	—	—	—	—	—	—
Newly diagnosed, n (%)	42 (36.5)	19 (31.7)	—	—	—	—	—	—
Thrombosis	80 (69.6)	25 (41.7)	—	—	—	—	—	—
Venous thrombosis, n (%)	38 (33.0)	17 (28.3)	—	—	—	—	—	—
DVT, n (%)	31 (27.0)	13 (21.7)	—	—	—	—	—	—
PE, n (%)	16 (13.9)	5 (8.3)	—	—	—	—	—	—
Arterial thrombosis, n (%)	61 (53.0)	15 (25.0)	—	—	—	—	—	—
Stroke, n (%)	30 (26.1)	6 (10.0)	—	—	—	—	—	—
CAD, n (%)	10 (8.7)	0	—	—	—	—	—	—
Both venous and arterial thrombosis, n (%)	19 (16.5)	7 (11.7)	—	—	—	—	—	—
Pregnancy morbidity, n (%)	42/94 (44.7)	43/55 (78.2)	—	—	—	—	—	—
Both thrombosis and pregnancy morbidity, n (%)	7/94 (7.4)	8/55 (14.5)	—	—	—	—	—	—
aCL (+), n (%)	29 (25.2)	0	0	0	0	1 (3.8)	0	0
aβ2GPI (+), n (%)	62 (53.9)	0	0	0	1 (2.4)	0	1 (5.9)	1 (2.0)
LA (+), n (%)	96 (83.5)	0	0	0	7 (16.7)	0	1 (5.9)	0
Double positive aPLs, n (%)	30 (26.1)	0	—	—	—	—	—	—
Triple positive aPLs, n (%)	21 (18.3)	0	—	—	—	—	—	—

SPAPS, seropositive antiphospholipid syndrome; SNAPS, seronegative antiphospholipid syndrome; OA, osteoarthritis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; AS, ankylosing spondylitis; DVT, deep venous thrombosis; PE, pulmonary embolism; AT, arterial thrombosis; CAD, coronary atherosclerotic heart disease; LA, lupus anticoagulant; aPLs, antiphospholipid antibodies.

Association between aPLs and clinical manifestations

Compared to the APS patients only with a history of pregnancy morbidity, the positivity and levels of aβ2GPI, aPS/PT, aPS IgG and APhL IgG were significantly increased in APS patients only with a history of thrombosis (Table 4 and Supplementary Figure 2). Furthermore, the prevalence of LA in APS patients with a history of thrombosis only was significantly higher than in APS patients with a history of pregnancy morbidity only (Table 4).

Presence of thrombosis was significantly associated with aPS/PT [odds ratio (OR) 2.48, 95% confidence interval (CI) 1.32-4.69, $p = 0.004$], aPS IgG (OR 1.90, 95%CI 1.01-3.60, $p = 0.046$), and APhL IgG (OR 2.26, 95%CI 1.18-4.34, $p = 0.013$). Arterial thrombosis was significantly associated with aPS/PT (OR 2.28, 95%CI 1.24-4.20, $p = 0.008$), aPS IgG (OR 2.58, 95% CI 1.38-4.82, $p = 0.003$), and APhL IgG (OR 2.54, 95%CI 1.36-

TABLE 2 Prevalence of non-criteria antibodies.

	SPAPS (N=115)	SNAPS (N=60)	Disease controls (N=122)	Healthy controls (N=50)	p_1[a]	p_2[a]
aAnnexinV, n (%)	77 (67.0)	26 (43.3)	68 (55.7)	8 (16.0)	0.000	0.904
aPE, n (%)	47 (40.9)	10 (16.7)	15 (12.3)	5 (10.0)	0.000	0.317
aPS/PT, n (%)	69 (60.0)	10 (16.7)	8 (6.6)	1 (2.0)	0.000	0.005
aPS IgG, n (%)	62 (53.9)	9 (15.0)	19 (15.6)	0	0.000	0.418
aPS IgM, n (%)	23 (20.0)	6 (10.0)	7 (5.7)	1 (2.0)	0.000	0.134
APhL IgG, n (%)	64 (55.7)	4 (6.7)	8 (6.6)	0	0.000	0.550
APhL IgM, n (%)	22 (19.1)	13 (21.7)	8 (6.6)	1 (2.0)	0.000	0.000

p_1, p-values refer to SPAPS vs. HC and DC; p_2, p-values refer to SNAPS vs. HC and DC; SPAPS, seropositive antiphospholipid syndrome; SNAPS, seronegative antiphospholipid syndrome; DC, disease control; HC, healthy control. [a]Pearson Chi-square test.

TABLE 3 Diagnostic values of criteria and non-criteria antibodies.

	Sensitivity (%)	Specificity (%)	Accuracy (%)	Youden Index	PPV (%)	NPV (%)	OR (95%CI)	PLR	NLR
aCL	16.57	99.42	57.64	0.1599	96.67	53.94	33.97 (4.57, 252.40)	28.50	0.84
aβ2 GPI	34.29	98.26	65.99	0.3254	95.24	59.51	29.39 (9.00, 95.98)	19.66	0.67
LA	54.86	95.35	74.93	0.5021	92.31	67.49	24.91 (11.54, 53.78)	11.79	0.47
APhL IgG	38.86	95.35	66.86	0.3421	89.47	60.52	13.03 (6.02, 28.19)	8.35	0.64
aPS IgM	16.57	95.35	55.62	0.1192	78.38	52.90	4.07 (1.80, 9.19)	3.56	0.88
aPS/PT	45.14	94.77	69.74	0.3991	89.77	62.93	14.90 (7.15, 31.06)	8.63	0.58
APhL IgM	20.00	94.77	57.06	0.1477	79.55	53.79	4.53 (2.10, 9.75)	3.82	0.84
aPS IgG	41.04	88.95	64.93	0.2999	78.89	60.00	5.61 (3.19, 9.86)	3.72	0.66
aPE	32.57	88.37	60.23	0.2094	74.03	56.30	3.67 (2.09, 6.45)	2.80	0.76
aAnnexinV	58.86	55.81	57.35	0.1467	57.54	57.14	1.81 (1.18, 2.77)	1.33	0.74

PPV, positive predictive value; NPV, negative predictive value; OR, odds ratio; PLR, positive likelihood ratio; NLR, negative likelihood ratio; CI, confidence interval.

4.75, p = 0.003). Additionally, stroke was significantly associated with aPS/PT (OR 2.23, 95%CI 1.05-4.73, p = 0.034) and aPS IgG (OR 2.44, 95%CI 1.13-5.24, p = 0.020).

Added values of different aPLs in the diagnosis of APS and SNAPS

As shown in Table 2, the prevalence of aAnnexinV, aPE, aPS/PT, aPS IgG, aPS IgM, APhL IgG, and APhL IgM in SNAPS patients were 43.3%, 16.7%, 16.7%, 15.0%, 10.0%, 6.7%, and 21.7%, respectively. By adding the "non-criteria" aPLs, the aPL positive rate was increased from 65.7% (criteria aPLs only) to 87.4% in APS patients (Supplementary Figure 3).

Seven single antibodies or two to five antibody combinations were analyzed among SNAPS patients, respectively. The ROC curves were applied to evaluate the predictive value, and the ones with the highest AUC values were shown in Figure 2 and Supplementary Table 7. The APhL IgG showed the highest AUC of 0.597 among single non-criteria aPLs in SNAPS patients. The APhL IgG/IgM showed the highest AUC of 0.694 among two antibody combinations. The APhL IgG/IgM plus aAnnexinV showed the highest AUC of 0.708 among three antibody

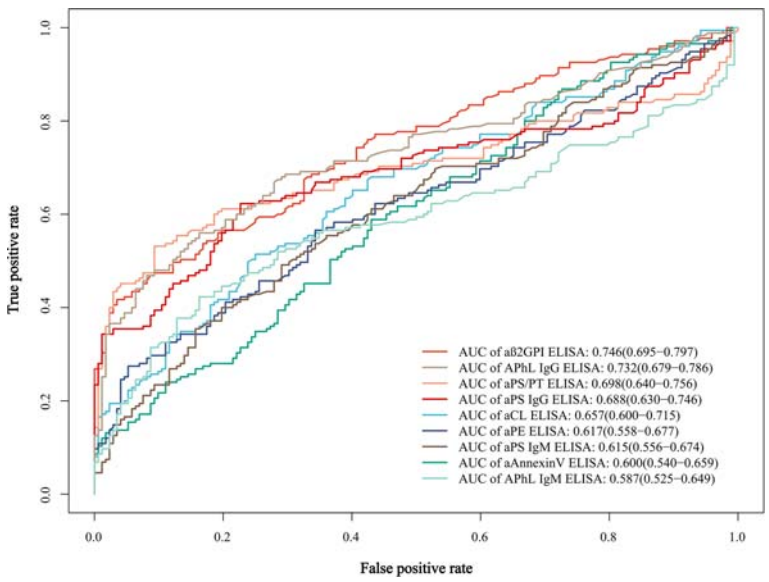

FIGURE 1
Receiver operating characteristic (ROC) curves and area under the curve (AUC) of the single aPLs. AUC values of each curve are shown with 95% confidence interval (CI) and are listed in a descending order.

TABLE 4 Prevalence of different antibodies among APS patients with a history of thrombosis only, pregnancy morbidity only, or both.

	Thrombosis only (N=90)	Pregnancy morbidity only (N=70)	Both (N=15)	p_1 [a]	p_2	p_3
aCL, n (%)	19 (21.1)	9 (12.9)	1 (6.7)	0.173	0.683[b]	0.293[b]
aβ2 GPI, n (%)	45 (50.0)	14 (20.0)	3 (20.0)	0.000*	1.000[b]	0.048[b,*]
LA, n (%)	59 (65.6)	31 (44.3)	6 (40.0)	0.007*	0.761[a]	0.059[a]
aAnnexinV, n (%)	52 (57.8)	41 (58.6)	10 (66.7)	0.920	0.561[a]	0.517[a]
aPE, n (%)	36 (40.0)	18 (25.7)	3 (20.0)	0.058	0.753[b]	0.161[b]
aPS/PT, n (%)	51 (56.7)	22 (31.4)	6 (40.0)	0.001*	0.522[a]	0.230[a]
aPS IgG, n (%)	45 (50.0)	22 (31.4)	4 (26.7)	0.015*	1.000[b]	0.156[b]
aPS IgM, n (%)	15 (16.7)	12 (17.1)	2 (13.3)	0.936	1.000[b]	1.000[b]
APhL IgG, n (%)	45 (50.0)	19 (27.1)	4 (26.7)	0.003*	1.000[b]	0.161[b]
APhL IgM, n (%)	16 (17.8)	18 (25.7)	1 (6.7)	0.223	0.172[b]	0.456[b]

p_1, p-values refer to Thrombosis vs. Pregnancy morbidity; p_2, p-values refer to Both vs. Pregnancy morbidity; p_3, p-values refer to Thrombosis vs. Both. [a]Pearson Chi-square test; [b]Fisher's exact test. *$p < 0.05$.

combinations. The APhL IgG/IgM, aPS IgG plus aPE showed the highest AUC of 0.715 among four antibody combinations. The APhL IgG/IgM, aPS IgG, aPE plus aAnnexinV showed the highest AUC of 0.720 among five antibody combinations.

Discussion

This study assessed the clinical significance of non-criteria aPLs in APS. Among these non-criteria aPLs, aAnnexin V showed the highest sensitivity, while APhL and aPS IgM showed the highest specificity. APhL, aPS/PT, and aPS IgG may be potential biomarkers to predict thrombotic risk in APS.

It has been reported that aPS/PT was a useful diagnostic marker for thrombosis in APS (22), especially for arterial thrombosis. Among 323 patients with or without APS who tested for aPLs, aPS/PT could additionally identify 2% of obstetric patients and 3% of thrombotic patients (17). The positive rate of aPS/PT was 16.7% in SNAPS in our study, it is also a valuable marker for SNAPS (13), and could additionally identify 9% of obstetric patients and 5% of thrombotic patients

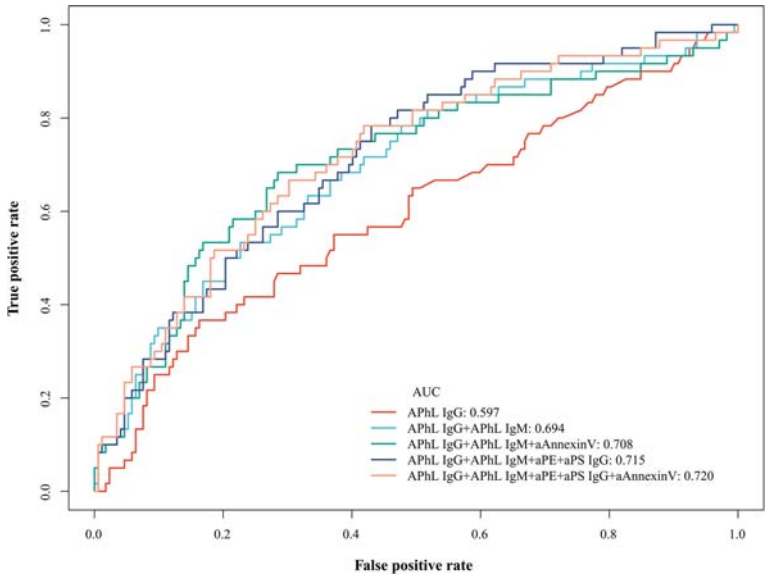

FIGURE 2
Receiver operating characteristic (ROC) curves and area under the curve (AUC) of single or combined non-criteria antibodies among SNAPS patients and controls. In seven single antibodies and combinations of two to five antibodies, the ROC curves with the highest AUC values are shown. Among SNAPS patients, the APhL IgG showed the highest AUC value in single antibodies. The APhL IgG/IgM showed the highest AUC among two antibody combinations, and the same is true of the other three combinations.

among patients with APS. Therefore, aPS/PT can be used as a diagnostic marker in APS and may also indicate thrombosis.

Annexin V is a potent anticoagulant protein by its ability to bind phospholipids, form crystals and block the availability of phospholipids to phospholipid-dependent coagulation enzymes (23, 24). According to Pooled Data from three studies, Annexin V resistance was present in more than half of patients with APS (9). And the aPL-mediated reduction of Annexin V has been observed on placental trophoblasts (25) and endothelial cells (26–28). The presence of aAnnexinV may impair the anticoagulant shield and lead to thrombosis and pregnancy morbidity. Although aAnnexinV might be involved in the pathogenesis of APS, it appeared that conflicting conclusions were observed between aAnnexinV and clinical features (16, 29 , 23, 30–33). In our study, the clinical significance of aAnnexinV is not as predictive as other non-criteria aPLs. Still, combinations of aAnnexinV and other non-criteria antibodies may better recognize patients with seronegative APS.

This study evaluated the clinical significance of antibodies against phospholipid antigens, including aPE, aPS, and APhL. The antibodies against phospholipid antigens include β2-GPI-dependent (β2-GPI-dependent) and β2-GPI-independent forms. The aPLs in the serum of patients with infectious diseases are β2-GPI-independent, which are unrelated to thrombosis (34). The β2-GPI-dependent aPLs were more specific to APS. Thus, β2-GPI was a cofactor for aPE (12) and aPS (35, 36). There were no associations between aPE and clinical manifestations in our and other APS cohorts (37, 38). Therefore, aPE may not serve as a marker for thrombosis or pregnancy morbidity in APS. The diagnostic value of aPS revealed high sensitivity and specificity (35), and it was associated with thrombosis. Therefore, aPS may serve as a diagnostic indicator for APS.

APhL reduced the false positives associated with the aCL test and improved the specificity in diagnosing APS (39). The prevalence of APhL was 11.5% and the specificity was 92.8 to 97.6% (40). APhL was associated with arterial thrombosis and pregnancy-related morbidity (40). We confirmed the association between APhL IgG and arterial thrombosis. APhL IgG is also a promising biomarker for SNAPS.

The combined autoantibodies tests might help to increase the sensitivity in the diagnosis of APS, but decrease the specificity (16). In our study, the sensitivity increased to 87.4% after adding all these 7 non-criteria aPLs. The presence of any 7 or more aPLs was linked with arterial thrombosis with an odds ratio (OR) of 4.1 (41). A longitudinal study conducted for 15 years showed that the risk of thrombosis progressively increased with the number of positive aPLs (42). The risk of thrombosis increased to thirtyfold higher after adding 4 positive antibody tests (42). Patients with catastrophic APS, a severe APS, had the highest number of non-criteria aPLs (43). Therefore, aPL profiling is more important than single aPL tests in APS diagnosis and risk stratification.

In clinical practice, it is unlikely to test all non-criteria aPLs but reasonably only includes highly specific non-criteria aPLs. It will improve the diagnostic accuracy for APS. In this study, we demonstrated that the aPS/PT, aPS and APhL could be three promising markers for diagnosing APS.

This study has some limitations. First, we didn't include patients with recurrent thrombosis or pregnancy morbidity without APS as disease controls. Further studies are needed to evaluate the clinical significance of these aPLs. Second, the sample size of SNAPS patients was relatively small. This sample requires verification with a larger population for the diagnostic utility of non-criteria aPLs in SNAPS. Third, this is a single-center study. In the future, it will be worthwhile to initiate a multicenter investigation with a larger sample size to determine how consistently the non-criteria aPLs improve the diagnostic accuracy in APS.

Conclusions

Several non-criteria aPLs were significantly increased in patients with APS. These non-criteria aPLs could improve the diagnostic value for APS. Detecting aPS/PT, aPS, and APhL may serve as reliable markers to predict the risk of SNAPS and thrombosis.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the ethic committees of Peking University People's Hospital (2019PHB253). The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors were involved in drafting and revising the manuscript, and all authors approved the final version to be submitted for publication. XL and LZ collected and analyzed data. LZ, HL, QC, and FS performed the ELISA assays. XL, LZ, HL, ZY, YJ, CL, and JG performed the statistical analysis and wrote the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.972012/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Level of criteria and non-criteria antibodies in patients with antiphospholipid syndrome (APS) and in control subjects as determined by ELISA. Dot plot of the nine antibody titers among different diagnostic groups, with the lines showing the quartile values. *P*-values by the Mann-Whitney *U* test. SPAPS, seropositive antiphospholipid syndrome; SNAPS, seronegative antiphospholipid syndrome; OA, osteoarthritis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; AS, ankylosing spondylitis; HC, healthy controls. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; NS, not significant.

SUPPLEMENTARY FIGURE 2
Level of different antibodies in APS patients with a history of thrombosis only, pregnancy morbidity only, or both. Dot plot of the nine antibody titers among groups with different clinical manifestations, with the lines showing the quartile values. The *p*-values from the Mann-Whitney *U* test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; NS, not significant.

SUPPLEMENTARY FIGURE 3
Diagnostic values among criteria and non-criteria antibodies. By adding the "non-criteria" aPLs, the aPL positive rate was increased from 65.7% (criteria aPLs only) to 87.4% in APS patients.

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Low dose versus standard dose rituximab for the treatment of antiphospholipid syndrome: A pilot study from a tertiary medical center

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Background: To investigate the therapeutic effects and safety of low-dose and standard-dose rituximab (RTX) in the treatment of antiphospholipid syndrome (APS).

Methods: In this real-world study, we included 22 consecutive patients with APS who received RTX. Standard dose (SD) was defined as an overall dosage of RTX ≥ 1000mg in the induction period, and low dose (LD) was defined as an overall dosage of RTX <1000mg.

Results: Of included patients, 1 patients died, 2 patients withdrew and 19 patients completed 6-month follow-up. Nine patients received SD-RTX and 13 patients received LD-RTX, and elder patients [LD-RTX vs. SD-RTX: (49.1 ± 15.5) vs. (35.8 ± 12.3) years, $p = 0.044$] and patients with later-onset [LD-RTX vs. SD-RTX: (46.8 ± 16.3) vs. (31.3 ± 13.6) years, $p = 0.029$] were more frequently included in LD-RTX than SD-RTX. Following 6 month RTX treatment, 8 patients (42.1%) achieved complete remission, 8 patients (42.1%) achieved partial remission and 3 patients (15.8%) showed no remission. The titers of anticardiolipin antibodies [baseline vs. 6 months: 30.8 (10.7, 90) vs. 19.5 (2.45, 69.10) U/L, $p = 0.023$] and the levels of erythrocyte sedimentation rate [baseline vs. 6 months: 29 (6, 63) vs. '6 (3, 14) mm/h, $p = 0.021$] exhibited a significantly decrease in all APS patients. Remission rate and titers of anti-β2-glycoprotein I and lupus anticoagulant did not differ significantly between two groups.

Conclusion: RTX might be a safe and effective option for patients with APS, and low dose confers equal efficacy as standard dose. Further cohort studies are needed to confirm our findings.

KEYWORDS

rituximab, antiphospholipid syndrome, standard dose, low dose, real-world study

Introduction

Antiphospholipid syndrome (APS) is systemic autoimmune disease characterized by combination of vascular thrombosis, obstetrical complications and persistent presence of circulating antiphospholipid antibodies (aPLs) such as anti-β2-glycoprotein I (aβ2GPI), anticardiolipin antibodies (aCL) and lupus anticoagulant (LAC) (1). Up to now, the first-line treatment of APS consists of aspirin, low-molecular-weight heparin or warfarin. However, antithrombotic strategies are usually not effective for nonthrombotic manifestations, nephropathy and microthrombosis. On the basis of newly understood immunological mechanisms, immunomodulatory approaches targeting mTOR, B cells, and complement have been proposed as an add-on treatment in APS patients (2, 3).

As B-cells play an important role in APS pathophysiology, elimination of B cells could be a promising treatment option. Rituximab (RTX) is an anti-CD20 monoclonal antibody, which ultimately results in B-cell depletion and dysfunction (4). Although there are no controlled studies to compare the efficacy between RTX and placebo, the experience of RTX in the treatment APS has been proven by some observational studies in the last few years (5–8). For example, Doruk E. et al. and Sciascia S. et al. found RTX may be effective in controlling some non-criteria manifestations (thrombocytopenia, skin ulcer and cognitive dysfunction) regardless of substantial change in aPL profiles (8, 9). A multicentre Israeli study revealed complete response was associated with a decrease in aPL titers within 4–6 months after RTX treatment (7). To sum up, RTX may be an efficient treatment in APS, especially in controlling non-criteria manifestations.

Nevertheless, the safety of the long term and high dose RTX is the main concern. As we all know, the side effects of RTX included infections, allergy, infusion reaction, and so on (10). Recently, several studies have suggested that a low-dose regimen were closely similar to the successful results obtained with conventional regimens in other autoimmune diseases (10–14). In addition, a significantly lower incidence of infections and substantial costs saving were seen with low dose RTX (LD-RTX) compared with standard dose (12, 14). These outcomes indicated that high dose RTX might not be necessary for all the patients with autoimmune diseases and low dose RTX could be a promising new option with a satisfactory response rate.

However, the efficacy of low-dose RTX remains elusive in APS. The aim of our pilot study is to investigate the efficacy and safety of low-dose and standard-dose RTX for APS patients.

Methods

Patients

This is a real-world study in Department of Rheumatology and Immunology, Peking University People's Hospital, based on our dynamic retrospective cohort of APS between July 2009 and January 2021. The diagnosis of APS was confirmed by two rheumatologists (YZG and CL) according to the 2006 revised Sapporo criteria (15) or catastrophic antiphospholipid syndrome (CAPS) according to the current diagnostic criteria (16). Another inclusion criterion was disease onset age ≥ 18 years. This study was approved by the ethics committee of Peking University People's Hospital (2019PHB253-01) and complied with the Declaration of Helsinki guidelines. Written informed consent was obtained from each patient.

Clinical and laboratory data collection

Baseline data were obtained from the electronic medical records before the initial RTX treatment, including demographics, duration of symptoms, APS-related manifestations, laboratory assessment, and details of prior treatment. Patients were divided into two groups, a standard-dose RTX (SD-RTX) group which received a total of more than or equal to 1000mg in 4 weeks and a LD-RTX which received a total of less than 1000mg. LAC was measured by dilute Russell viper venom test (dRVVT) as previously described followed by mixing studies and confirmatory testing when prolonged. Generally, the first step is a sensitive coagulation (dRVVT), the next step is a mixing study and the final confirmatory test involves adding phospholipid, leading to, for example, the dRVVT confirm ratio (17). The titers of aCL (IgA/IgG/IgM)

were measured by enzyme linked immunosorbent assay (ELISA) (ORGENTEC, Germany, Product Number: ORG 515S). ACL IgG, IgM and IgA were measured by ELISA (EUROIMMUN, Germany, Product Number: EA 1621-9601 G for IgG, EA 1621-9601 M for IgM and EA 1621-9601 A for IgA). The titers of aβ2GPI (IgA/IgG/IgM) were also measured by ELISA (EUROIMMUN, Germany, Product Number: EA 1632-9601 P). Aβ2GPI IgG, IgM and IgA were measured by ELISA (EUROIMMUN, Germany, Product Number: EA 1632-9601 G for IgG, EA 1632-9601 M for IgM and EA 1632-9601 A for IgA). Venous thromboembolic events (e.g., deep venous thrombosis of the upper limbs of the legs, visceral venous thrombosis, and/or pulmonary embolism) were confirmed by limb ultrasound, pulmonary computed tomography (CT) or scintigraphy (ventilation/perfusion), abdominal pelvic CT scan and vessel angiography as indicated. Arterial thrombotic events (e.g., peripheral arterial thrombosis, acute cerebral infarction, and/or visceral arterial thrombosis) were diagnosed using typical clinical pictures with positive arteriography [e.g., leg or upper limb ultrasound, CT, or magnetic resonance angiography (MRA)] and surgery. The adjusted global antiphospholipid syndrome score (aGAPSS) was calculated for each patient by adding the points corresponding to the risk factors, excluding antibodies to phosphatidylserine/prothrombin (aPS/PT) that are not routinely tested in most clinical laboratories, as previously described (18). The aGAPSS ranged from 0 to 17.

Follow-up procedure and clinical outcomes

All patients were prospectively followed up after initial RTX administration at month 3 and 6 by the same medical team (YZG and CL). Patients with APS typically require lifelong warfarin anticoagulation following a thrombotic event due to a significant risk of recurrent thrombosis. International normalized ratio (INR) is the preferred test of choice for patients taking warfarin anticoagulant therapy. The INR target is between 2.0 and 3.0 in patients with APS, according to the anticoagulation guidelines of the American College of Chest Physicians (19). Response was evaluated 3 and 6 months after the first dose of RTX. Follow-up information was also obtained from electronic medical records and regular medical examination reports. In accordance with the revised Sapporo criteria (15), complete response (CR) was defined as achieving full resolution of the "indicated manifestation"; partial response (PR) was defined as a favorable response occurred but did not meet the criteria for complete response. Overall response included complete response and partial response. Patients who did not reach remission were considered non-responders (NR). For thrombocytopenia, complete response was defined as a platelet count of > 100×10^9/L, partial response as $80–100\times10^9$/L, and no response as < 80×10^9/L. For cardiac manifestations, complete response was defined as the disappearance of echocardiographic lesions, partial response as 50% improvement of echocardiographic lesions, and no response as no change or worsening of echocardiographic lesions. For skin ulcer, complete response was defined as disappearance by physical examination and digital imaging, partial response as > 50% improvement, and no response as no change or worsening of skin ulcers. For cognitive dysfunction, complete remission was defined as normalization of the cognitive impairment index with 50% improvement, partial response as abnormal cognitive impairment index with 50% improvement, and no response as no change or worsening of the cognitive impairment index. Adverse events associated with RTX were assessed during drug infusion and throughout follow-up. All adverse events were graded according to the Common Terminology Criteriafor Adverse Events, version 5 (CTCAE) (20).

Statistical analysis

All statistical analyses were performed using Statistical Product and Service Solutions (SPSS) 25.0 for Windows (IBM, New York, USA). GraphPad Prism version 8.0 were used to produce the graphs. The data were expressed as percentages for categorical variables, mean ± standard deviation (SD) for normally distributed continuous variables, and median [interquartile range (IQR)] for skewedly distributed continuous variables. Differences between LD group and SD group were analyzed by chi-square test or Fisher's exact as appropriate for categorical variables, and two-tailed independent-sample t test or Mann-Whitney U-test for continuous variables. Differences between LD group and SD group for laboratory manifestations were performed using Kruskal–Wallis tests at baseline and after 3 and 6 months. The cumulative probability of complete response of patients with different treatment dosage groups were drawn using the Kaplan–Meier method. Two-sided $p < 0.05$ was considered statistically significant.

Results

Study population and clinical characteristics at baseline

A total of 239 patients with thrombotic APS were enrolled in our cohort, and 22 patients with APS who received RTX as induction therapy. A flow diagram of the individuals at each stage was shown in Figure 1. There were a total of 143 courses of RTX. All patients did not receive additional immunosuppressants. The detailed clinical profiles were shown in Table 1.

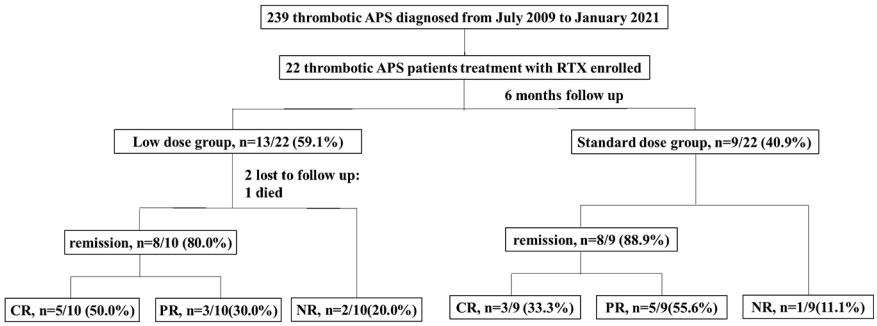

FIGURE 1
Flow chart of the patients with isolated thrombotic APS receiving rituximab therapy. There were 22 patients enrolled, with 13 patients received low dose rituximab (an overall dosage <1000mg) and 9 patients received standard dose rituximab (an overall dosage ≥1000mg). One patient discontinued treatment because of died, 2 patients lost to follow up, and 19 patients complement within 6-month follow up. The remission rate was 80.0% (50.0% CR, 30.0%PR) in the low dose group and 88.9% (33.3% % CR, 55.6%PR) in the standard dose group. CR, complete remission; PR, partial remission; NR, no remission.

TABLE 1 Detailed clinical profiles of APS patients with rituximab.

No.	Age of onset/ gender	aPL profile	Types	Anticoagulation therapy	Non-criteria manifestations	Initial induction therapy	RTX dose (mg)	Maintenance regimen	Outcomes at 6 months
1	45/F	aβ2-GPI, LAC	SAPS	No[&]	Thrombocytopenia	No	2000	Pre	NR
2	72/F	Triple positive	SAPS	Warfarin	Thrombocytopenia	Yes	800	Pre+HCQ	CR
3	24/F	Triple positive	SAPS	Warfarin	Thrombocytopenia	Yes	800	Pre+HCQ	CR
4	30/F	Triple positive	SAPS	No[&]	Thrombocytopenia	No	800	Pre+TAC	NR
5	31/F	LAC	SAPS	Warfarin, INR 1.5-2.0	Thrombocytopenia	No	800	CSA	PR
6	36/F	LAC	SAPS	Warfarin, INR 2.0-3.0	Thrombocytopenia	No	1000	MMF	CR
7	49/M	LAC	CAPS	No[&]	Thrombocytopenia	Yes	600	CSA	NR
8	55/M	aβ2GPI	SAPS	NA*	Thrombocytopenia	No	100	NA	NA*
9	70/F	aβ2GPI	SAPS	NA*	Thrombocytopenia	No	300	NA	NA*
10	24/M	aβ2-GPI, LAC	SAPS	Warfarin, INR 2.0-3.0	Thrombocytopenia	Yes	2000	MMF	CR
11	13/F	Triple positive	SAPS	Warfarin	Valvular vegetation	Yes	1000	NA	PR
12	41/F	Triple positive	PAPS	Warfarin, INR 2.0-3.0	Thrombocytopenia	Yes	800	RTX	CR
13	19/M	Triple positive	SAPS	Warfarin, INR 2.0-3.0	Thrombocytopenia, skin ulcer	Yes	1700	RTX	CR
14	53/F	aCL, LAC	SAPS	Warfarin, INR 1.5-2.0	Thrombocytopenia	No	1000	sirolimus	PR
15	49/M	Triple positive	CAPS	Warfarin, INR 2.0-3.0	Thrombocytopenia	No	500	MMF	CR
16	18/F	Triple positive	PAPS	Warfarin, INR 2.0-3.0	/	No	1000	AZA	PR

(Continued)

TABLE 1 Continued

No.	Age of onset/ gender	aPL profile	Types	Anticoagulation therapy	Non-criteria manifestations	Initial induction therapy	RTX dose (mg)	Maintenance regimen	Outcomes at 6 months
17	58/F	Triple positive	SAPS	Warfarin, INR 2.0-3.0	Thrombocytopenia	No	800	CSA	CR
18	43/M	Triple positive	SAPS	LMWH	Cognitive dysfunction	No	800	MMF	PR
19	25/M	aCL	PAPS	Warfarin, INR 2.0-3.0	Skin ulcer	No	600	None	PR
20	35/M	Triple positive	SAPS	Warfarin, INR 2.0-3.0	Thrombocytopenia	Yes	2000	RTX	PR
21	39/F	Triple positive	SAPS	Warfarin, INR 2.0-3.0	/	Yes	1000	MMF	PR
22	62/F	Triple positive	SAPS	NA*	Thrombocytopenia	No	100	CSA	Died

APS, antiphospholipid syndrome; aβ2GPI, anti-β2-glycoprotein I antibody; aCL, anticardiolipin antibody; LAC, lupus anticoagulant; PAPS, primary antiphospholipid syndrome; SAPS, secondary antiphospholipid syndrome; CAPS, catastrophic antiphospholipid syndrome; Pre, prednisone; RTX, Rituximab; HCQ, hydroxychloroquine; TAC, tacrolimus; CSA, Cyclosporin; MMF, mycophenolate mofetil; AZA, azathioprine; NA, Not available due to lost to follow-up. CR, complete remission, PR, partial remission; NR, no remission; LMWH, low molecular weight heparin.
*Patients lost to follow-up.
&Patients did not receive anticoagulation therapy due to sustained severe thrombocytopenia ($<20\times10^9$/L).

Of 22 patients with APS who received RTX, 13 patients treated with LD-RTX and 9 patients treated with SD-RTX. All patients received hydroxychloroquine during RTX induction therapy. Of 19 patients completed 6-month follow-up, 3 patients did not receive anticoagulation therapy due to sustained severe thrombocytopenia, one patient received LMWH, and 15 patients received warfarin. Of those 15 patients received warfarin, 2 patients did not achieved target INR because of thrombocytopenia ($30\text{-}50\times10^9$/L). The clinical and laboratory characteristics of the patients at baseline were presented in Table 2. Patients included in LD-RTX had the characteristics of older age [(49.1 ± 15.5) vs. (35.8 ± 12.3) years, $p = 0.044$] and later-onset [(46.8 ± 16.3) vs. (31.3 ± 13.6) years, $p = 0.029$] than SD-RTX. We next adopted logistics models to test whether there was a relationship between age or age of onset and the dose of RTX, and found there was no significant relationship between age and dose of RTX [OR 1.091 (0.592 - 2.011), p=0.087] or age of onset [OR=0.794 (0.465 - 1.354), p=0.397]. Gender, clinical or laboratory features, aGAPSS scores and the percentage of RTX usage as initial induction treatment did not show any significant differences between the two groups.

Comparison of treatment response between standard - and low-dose RTX groups at 6 months

Follow-up data were available for 20 patients (90.1%), and one of them died within 1 month. Of 19 patients who completed 6-month follow-up, 8 patients (42.1%) achieved complete response. Following rituximab treatment, the levels of aPLs, immunoglobulin, C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were assessed at 3 months and 6 months post therapy (Figure S1). None of the above parameters showed significant changes after 3 months. After 6 months, a significant decrease of aCL titers {including aCL IgA/IgG/IgM (U/L) [30.8 (10.7,90) vs. 19.50 (2.45,69.10), p=0.023] and aCL IgG (U/L) [75.39 (4.98,120) vs. 54.21(2,73.04), p=0.043]} and ESR (mm/h) [29 (6, 63) vs. 6 (3, 14), p=0.021] was observed, and other parameters, including aβ2-GPI, LAC, immunoglobulin and CRP did not show any significant decrease (Table 3).

Cumulative CR rates were compared between the two groups (Figure 2A). There was no significant difference in CR rate for 6 months between the groups (SD-RTX 33.3% vs. LD-RTX 50%, log-rank, $p = 0.807$). We also compared the numbers of patients with CR, PR and NR (Figure 2B and Table S1) and laboratory parameters (Table 4) between the two groups. Apart from the levels of IgA were significantly lower in LD-RTX group than SD-RTX group [(1.95 ± 0.26) vs. (2.80 ± 0.21) g/L, p=0.03], there were no significant differences in different status of remission rate and laboratory parameters between SD-RTX group and LD-RTX group at 3 months and 6 months.

Safety and adverse reactions

Adverse events during 6 month after RTX initiation were summarized in Table 5. In our cohort, serious adverse events were not reported. There were no significant differences between the two groups in the number of adverse events. In LD-RTX group, 1 patient developed pulmonary infection and 1 patient

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TABLE 2 Baseline characteristics of APS patients with LD-RTX versus SD-RTX.

Variables	Total (n = 22)	Low dose (n = 13)	Standard dose (n = 9)	*p*
Gender (M/F)	8/14	5/8	3/6	1
Age, years	43.6 ± 15.5	49.1 ± 15.5	35.8 ± 12.3	**0.044**
Age of onset, years	40.5 ± 16.8	46.8 ± 16.3	31.3 ± 13.6	**0.029**
Clinical criteria manifestation				
Venous thrombosis, n (%)	14 (63.6)	7 (53.8)	7 (77.8)	0.38
Artery thrombosis, n (%),	12 (54.5)	7 (53.8)	5 (55.6)	1
Arteriovenous thrombosis, n (%)	4 (18.2)	1 (7.7)	3 (33.3)	0.264
Laboratory criteria manifestation				
aβ2 GPI (IgA/IgG/IgM) (RU/mL)	109.9 ± 96.2	117.7 ± 91.5	100.3 ± 106.3	0.699
aβ2-GPI IgA (n=13) (RU/mL)	7.13 (2.07, 43.82)	2.42 (2.04, 104.38)*	10.93 (3.06, 50.34)[&]	0.418
aβ2-GPI IgG (n=13) (RU/mL)	16.92 (3.92, 92.70)	15.28 (3.85, 71.56)*	23.89 (3.63, 120.07)[&]	0.608
aβ2-GPI IgM (n=13) (RU/mL)	17.24 (2.43, 52.46)	41.33 (9.62, 71.03)*	8.21 (2.37, 35.97)[&]	0.341
aβ2 GPI +, n (%)	16 (76.2)	10 (83.3)	6 (66.7)	0.611
aCL (IgA/IgG/IgM) (U/L)	30.8 (13.8, 90.0)	34.9 (14.1, 84.7)	17.3 (13.8, 90.0)	0.943
aCL IgA (n=13) (U/L)	2 (2, 10.64)	2 (2, 61)*	4.76 (2, 10.79)[&]	0.504
aCL IgG (n=13) (U/L)	56.05 (4.98, 100.9)	56.06 (13.76, 97.70)*	61.25 (3.51, 110.45)[&]	0.941
aCL IgM (n=13) (U/L)	2.32 (2, 7.81)	2.2 (2, 8.65)*	3.65 (2.05, 6.63)[&]	0.824
aCL + (n, %)	15 (71.4)	8 (66.7)	7 (77.8)	0.659
dRVVT screen (s)	56.9 (41.6, 101.7)	54.6 (42.9, 72.3)	83.6 (40.0, 128.6)	0.152
LAC	1.6 (1.4, 2.1)	1.6 (1.4, 1.9)	1.9 (1.4, 2.6)	0.342
LAC+, n (%)	18 (90.0)	10 (90.9)	8 (88.9)	1
Triple positive aPLs, n (%)	13 (59.1)	8 (61.5)	5 (55.6)	1
Laboratory manifestations				
White blood cell count ($\times10^9$/L)	6.0 (4.2, 9.1)	5.7 (4.8, 9.1)	6.3 (4.0, 6.5)	0.738
Lymphocyte ($\times10^9$/L)	1.1 ± 0.6	1.1 ± 0.7	1.1 ± 0.5	0.951
Neutrophils ($\times10^9$/L)	4.1 (2.8, 6.2)	4.1 (2.9, 6.5)	3.8 (2.8, 5.4)	0.664
Hemoglobin (g/L)	117.0 (106.2, 128.0)	116.0 (77.0, 128.0)	117.0 (117.0, 128.0)	0.216
Platelet ($\times10^9$/L)	66.0 (50.2, 152.0)	55.0 (50.0, 129.2)	104.0 (59.0, 156.0)	0.367
ESR (mm/h)	29.0 (8.0, 63.0)	28.0 (9.0, 75.0)	34.0 (5.0, 39.0)	0.79
CRP (mg/L)	2.5 (1.0, 4.4)	3.2 (2.5, 7.4)	1.0 (0.9, 2.8)	0.075
IgA (g/L)	1.8 (1.2, 3.1)	1.4 (1.1, 2.6)	2.5 (1.2, 4.7)	0.182
IgG (g/L)	12.0 (8.4, 16.8)	12.1 (8.3, 22.9)	11.9 (8.7, 14.6)	0.841
IgM (g/L)	1.1 (0.6, 1.5)	1.2 (0.7, 1.8)	0.6 (0.5, 1.0)	0.102
C3 (g/L)	0.7 (0.5, 0.8)	0.7 (0.5, 0.9)	0.7 (0.6, 0.8)	0.64
C4 (g/L)	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.422
ANA≥1:80, n (%)	15 (68.1)	10 (76.9)	5 (55.6)	0.276
$CD19^+B$	56.0 (18.0, 232.0)	20.0 (10.0, 289.0)	63.0 (45.5, 187.5)	0.683
aGAPSS	11.52± 3.70	12.17 ± 3.83	10.67± 3.54	0.371
Initial usage, n (%)	14 (63.6)	8 (61.5)	6 (66.7)	0.584

*Eight patients did not have the data of serum levels of subtypes of aCL and aβ2-GPI.
[&]One patients did not have the data of serum levels of subtypes of aCL and aβ2-GPI.
LD, low-dose; SD, standard dose; RTX, rituximab. APS, antiphospholipid syndrome; M, male; F, female; y, years; aβ2GPI, anti-β2-glycoprotein I antibody; aCL, anticardiolipin antibody; dRVVT, dilute Russell viper venom test; LAC, lupus anticoagulant; aPL, antiphospholipid antibody; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M; C3, Complement 3; C4, Complement 4; aGAPSS, adjusted Global Antiphospholipid Syndrome Score.
The bold values mean statistical significance.

present with elevated liver enzyme post therapy that resolved spontaneously. In SD-RTX group, infusion reactions were documented in 1 patient who were treated with anti-histamines and glucocorticoids with no need to change the protocol. One patient died of macrophage activation syndrome.

Discussion

In this study, we found the levels of aCL and ESR decreased significantly after RTX treatment, and there were no significant differences in CR rate, aβ2GPI titers, LAC or adverse events

TABLE 3 Profiles of laboratory parameters following rituximab treatment.

Parameters	Pre-treatment	Post-treatment (n=19)			
		3 months		6 months	
		Value	p^*	Value	p^*
aβ2 GPI (IgA/IgG/IgM) (RU/mL)	76.27 (17.07,189.10)	137.13 (37.03, 267.10)	0.500	13.66 (3.46,132.49)	0.084
aβ2-GPI IgA (RU/mL)	6.01 (2.04,32)	101.04 (1.55,151.26)[&]	0.180	11.74 (2.33,73.07)[&]	0.893
aβ2-GPI IgG (RU/mL)	15.28 (5.12,141.50)	57.67 (4.28,83.49)[&]	0.655	23.89 (2,44.04)[&]	0.225
aβ2-GPI IgM (RU/mL)	40.92 (12.55,100.36)	29.29 (12.93,32.25)[&]	0.180	0.43 (2,25.23)[&]	0.225
aCL (IgA/IgG/IgM) (U/L)	30.8 (10.7,90)	30.10 (2.60,75.90)	0.735	19.50 (2.45,69.10)	**0.023**
aCL IgA (U/L)	2 (2,6.47)	61 (1.5,91.26)	0.317	4.76 (2,21.55)	0.655
aCL IgG (U/L)	75.39 (4.98,120)	72.76 (19.13,91.26)	0.665	54.21(2,73.04)	**0.043**
aCL IgM (U/L)	5.02 (2.16,9.76)	5.31 (1.5,7.71)	0.180	2.2 (2,5.88)	0.686
dRVVT screen (s)	56.9 (41.6, 101.7)	43.3 (40, 80.5)	0.612	56.3 (51.3, 75.1)	0.333
LAC	1.61 (1.37,2.17)	1.41 (1.12,2.15)	0.833	1.62 (1.49,2.06)	0.202
ESR (mm/h)	29 (6, 63)	14 (8, 23)	0.176	6 (3, 14)	**0.021**
CRP (mg/L)	2.49 (0.95,5.80)	1.68 (0.50,2.41)	0.674	1.51 (0.50,5.03)	0.678
IgA (g/L)	1.80 (1.15,3.37)	2.29 (1.42,3.86)	0.260	2.06 (1.37,3.85)	0.363
IgG (g/L)	12 (8.38,18.25)	11.20 (8.48,15.45)	0.173	10.45 (8.69,11.93)	0.140
IgM (g/L)	1.07 (0.52,1.54)	0.81 (0.37,1.54)	0.441	0.71 (0.31,1.24)	0.124
C3 (g/L)	0.67 (0.46,0.86)	0.78 (0.70,0.93)	0.173	0.98 (0.69,1.10)	0.177
C4 (g/L)	0.13 (0.10,0.16)	0.16 (0.12,0.20)	0.109	0.20 (0.15,0.24)	0.100
aGAPSS	11 (7, 13)	11 (7, 13)	0.344	8 (3.25,13.75)	0.107

[&]Six patients did not have the data of serum levels of subtypes of aCL and aβ2-GPI.
aβ2GPI, anti-β2-glycoprotein I antibody; aCL, anticardiolipin antibody; dRVVT, dilute Russell viper venom test; LAC, lupus anticoagulant; ESR, Erythrocyte sedimentation rate; CRP, C reactive protein; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M; C3, Complement 3; C4, Complement 4; aGAPSS, adjusted Global Antiphospholipid Syndrome Score.
*Comparison with parameters at baseline.
The bold values mean statistical significance.

for 6 months between patients taking low and standard doses of RTX for APS. Our findings suggested that low-dose treatment with RTX might be an alternative choice for elderly patients with APS as an induction therapy. To our knowledge, this is the first study to discuss the efficacy and safety of low-dose RTX in Asian patients with APS.

B cells, notably through aPLs production, play a key role in the development of APS (21). RTX is a chimeric monoclonal antibody targeting CD20 and can reduce cytokine secretion and autoantibody production by specifically targeting B cells expressing CD20 (22). Nowadays, RTX has been confirmed as an effective B cell depletion therapy in various autoimmune disorders, including APS (21, 22). Previous pilot studies have discovered favorable results of RTX in APS that a majority of APS patients achieved clinical improvement, especially non-criteria manifestations (8, 9, 23). A multicenter retrospective study revealed 55% refractory APS achieved CR following either 2 doses of 1000mg RTX (2 weeks apart) or 4 doses of 375mg/m^2 (once weekly) (7). Similarly, we found nearly half patients achieved complete remission after RTX during 6-month follow-up. Given all this evidence, application of RTX in APS contributes to decrease of aPLs and disease remission.

aPLs are mainly produced by plasma cells and circulating CD20 negative B cells (e.g. plasmablasts) (7, 24). Since RTX has no effect on memory B cells or long-lived plasma cells, the titers of aPLs might not show substantial change after RTX treatment, which has been proved by a phase II trial (8). However, our study revealed a significant decrease of aCL titers after RTX therapy, consistent with Ioannou Y et al's findings (25). Besides, Yang et al. found that after RTX, the titers of aCL decreased within 1-year follow-up and a substantial decrease of the titers of aβ2-GPI was observed within 2-year follow-up even after the recovery of B cells (6).These observations imply that different kinds of aPLs might have different mechanisms of production, and aCL might be mainly secreted by short-lived plasma cells in certain populations (24, 25). Moreover, some previous studies also showed significant decrease of aPL titers after RTX therapy (6, 7, 23). Apart from producing antibody directly, B cells have other immune functions, such as promoting antigen recognition and activation of T cells, or modulate immune response by secreting cytokines, which could also regulate autoantibody production indirectly. To sum up, it seems that at least for some patients, a decrease in aPL titres, within a certain period of time following rituximab treatment, is associated with a favorable outcome and

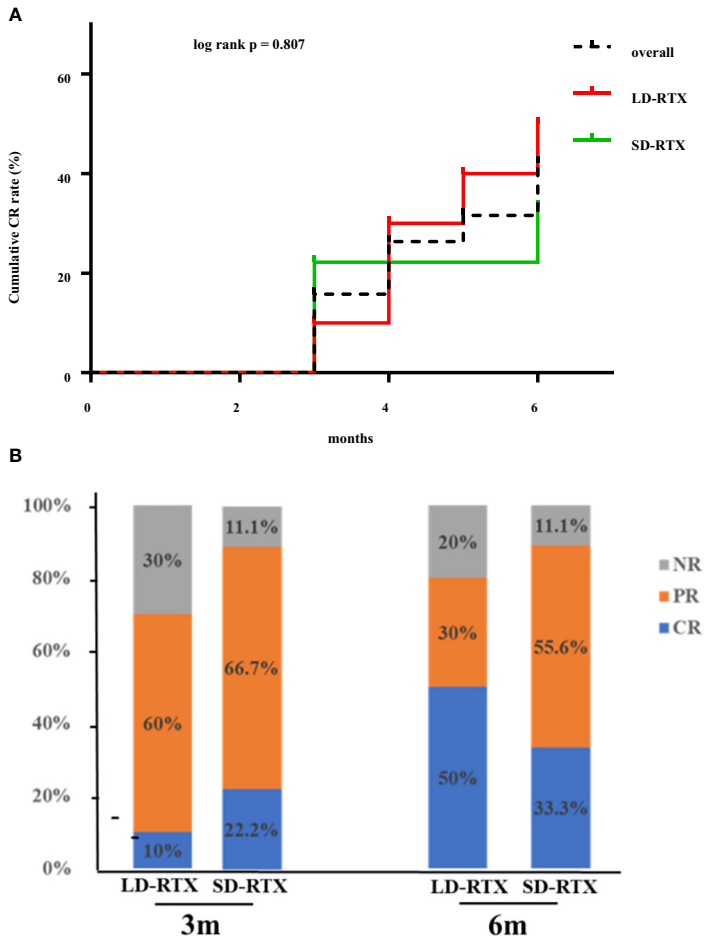

FIGURE 2
Remission rate at 6 months. **(A)** Cumulative complete remission rate for 6 months after induction therapy between patients with SD-RTX and LD-RTX. **(B)** Comparison of remission rate between patients with SD-RTX and LD-RTX at 3 months and 6 months. SD-RTX, standard-dose rituximab; LD-RTX, low-dose rituximab; CR, complete remission; PR, partial remission; NR, no remission.

TABLE 4 Comparison of laboratory manifestations in APS patients between low dose versus standard dose at 3 months and 6 months follow up.

Parameters	3 months follow up			6 months follow up		
	Low dose (n=10)	Standard dose (n=9)	*p*	Low dose (n=10)	Standard dose (n=9)	*p*
aβ2 GPI (IgA/IgG/IgM) (RU/mL)	121.61 ± 9.03	134.81 ± 4.63	0.417	87.56 ± 16.17	63.51 ± 15.67	0.317
aCL (IgA/IgG/IgM) (U/L)	25.66 ± 5.25	48.18 ± 6.12	0.068	0.75 ± 0.23	0.58 ± 0.22	0.613
dRVVT screen (s)	50.06 ± 4.57	59.08 ± 5.28	0.287	55.67 ± 24.62	81.13 ± 17.82	0.434
LAC	1.60 ± 0.16	1.53 ± 0.21	0.805	1.59 ± 0.29	1.92 ± 0.23	0.413
ESR (mm/h)	19.20 ± 7.92	14.18 ± 6.86	0.664	9.43 ± 4.81	10.05 ± 4.34	0.928
CRP (mg/L)	1.63 ± 0.61	4.08 ± 2.42	0.370	1.30 ± 1.42	1.86 ± 1.05	0.760
IgA (g/L)	2.76 ± 0.29	2.87 ± 0.20	0.772	1.95 ± 0.26	2.80 ± 0.21	**0.030**
IgG (g/L)	14.25 ± 2.20	11.89 ± 1.62	0.427	8.95 ± 0.98	11.46 ± 0.85	0.081
IgM (g/L)	1.19 ± 0.12	0.89 ± 0.11	0.135	1.11 ± 0.29	0.97 ± 0.41	0.785
C3 (g/L)	0.69 ± 0.05	0.80 ± 0.05	0.192	0.76 ± 0.09	1.01 ± 0.08	0.066
C4 (g/L)	0.12 ± 0.02	0.19 ± 0.02	0.082	0.16 ± 0.03	0.24 ± 0.03	0.079

aβ2GPI, anti-β2-glycoprotein I antibody; aCL, anticardiolipin antibody; LAC, lupus anticoagulant; ESR, Erythrocyte sedimentation rate; CRP, C reactive protein; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M; C3, Complement 3; C4, Complement 4; aGAPSS, adjusted Global Antiphospholipid Syndrome Score.
The bold values mean statistical significance.

TABLE 5 Adverse events at 6 months.

Variable	SD-RTX (n=9)	LD-RTX (n=11)*	*p*
Death, n (%)	0	1 (9.1)	0.45
Total number of selected events, n (%)	1 (11.1)	2 (18.2)	1
Cancer, n (%)	0	0	/
Infection, n (%)	0	1 (9.1)	0.45
Liver function impairment, n (%)	0	1 (9.1)	0.45
Infusion reaction, n (%)	1 (11.1)	0	0.45

SD-RTX, standard-dose rituximab; LD-RTX, low-dose rituximab.
*Two patients lost to follow-up.

may possibly be used as a marker for response to therapy. Other immune modulating mechanisms, independent of autoantibody production may also be associated with a clinical response to rituximab treatment. Therefore, different response of RTX treatment represents different populations, different methods of aPL detection or even different timing of aPL sampling.

Even if RTX has a relatively good safety profiles, immunosuppression, infusion reactions, and hepatitis virus/mycobacterial reactivations can occur (26). Besides, the B-cell total load in patients with APS is much less than that in patients with lymphoma. Furthermore, since RTX is expensive ($4912.79 in the USA or ¥7866.26 in PRC, per 500 mg) (14). Therefore, a reduced dosage of rituximab might still be sufficient for its therapeutic purpose with socioeconomically preference in preventing disease flare or suppressing disease activity.

In our study, we also found LD-RTX was an efficacious and safe treatment as SD-RTX for APS in decreasing titers of aPLs and reducing disease activity. LD-RTX has been shown similar efficacy to those successful results obtained with standard-dose regimens in autoimmune diseases (14, 27–29). For example, in rheumatoid arthritis (RA), Chatzidionysiou K et al. reported no significant difference was seen in the percentages of patients who achieved a European League Against Rheumatism good response at 6 months between high- (two doses of 1000 mg) and low-dose RTX groups (two doses of 500 mg) (29). In addition, in our study cohort, we found elder patients with APS tended to receive LD-RTX as induction therapy. Such tendency is also found in RA and ANCA-associated vasculitis (AAV) (14, 29). Furthermore the pharmacokinetics of RTX was highly variable among patients with AAV despite a dosing protocol that adjusted for the body surface area, and higher RTX exposure was not associated with important clinical outcomes (30). All these findings support our findings that SD-RTX might not be necessary for all the APS patients and some could be treated with LD-RTX, especially elder patients.

Study limitation

This study has several limitations. Firstly, due to the retrospective design of a single-center study, only patients who could be observed for more than 6 months were enrolled, which induced a degree of selection bias. Besides, T cell and B cell counts are not routinely tested in our center, thus we could not discuss the different effect on B cell depletion between LD-RTX and SD-RTX. Future prospective or multicenter studies are desired to validate our findings.

Conclusion

In the present study, we found that RTX might be effective in reducing aPL production and controlling disease activity, and LD-RTX may be as efficacious as SD-RTX in induction therapy for APS.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Peking University People's Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YG and XZ: data interpretation and analysis, writing of the original draft, review, and editing. GL and YZ: clinical data collection. HY: editing and follow-up of participants. CL: conceptualization, methodology, investigation, resources, data curation, supervision, manuscript editing, and funding

acquisition. The authors have read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.971366/full#supplementary-material

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